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CONTENTS OF VOLUME XXVII.

Funk, Casimir. The study of certain dietary conditions bearing on the problem of growth in rats ... 1
Wells, C. A., and Ewing, P. V. Cottonseed meal as an incomplete food. 15
Palmer, Leroy S. The physiological relation of plant carotinoids to the carotinoids of the cow, horse, sheep, goat, pig, and hen ... 27
McCollum, E. V., Simmonds, N., and Pitz, W. The relation of the unidentified dietary factors, the fat-soluble *A*, and water-soluble *B*, of the diet to the growth-promoting properties of milk ... 33
Ross, Ellison L. The influence of ether anesthesia on amino-acids of blood serum ... 45
Funk, Casimir, and Macallum, Archibald Bruce. Studies on growth. III. The comparative value of lard and butter fat in growth ... 51
Funk, Casimir, and Macallum, Archibald Bruce. Studies on growth. IV. The action of yeast fractions on the growth of rats ... 63
Roxas, M. L. The reaction between amino-acids and carbohydrates as a probable cause of humin formation ... 71
Sumner, James B. The direct determination of urea and ammonia in muscle ... 95
Bodansky, A. The chymase of *Solanum elaeagnifolium*. A preliminary note ... 103
Bloor, W. R., and Knudson, Arthur. The separate determination of cholesterol and cholesterol esters in small amounts of blood ... 107
Eddy, Walter H. The isolation of a growth-producing substance from sheep pancreas ... 113
Underhill, Frank P. Studies in creatine metabolism. I. Possible interrelations between acidosis and creatine elimination ... 127
Underhill, Frank P. Studies in creatine metabolism. II. The influence of alkali upon creatine elimination during inanition ... 141
Underhill, Frank P., and Baumann, Emil J. Studies in creatine metabolism. III. The influence of alkali upon the creatinuria of phlorhizin glycosuria ... 147
Underhill, Frank P., and Baumann, Emil J. Studies in creatine metabolism. IV. The relationship of creatinuria to carbohydrate metabolism and acidosis ... 151
Underhill, Frank P., and Bogert, L. Jean. Alterations in the output of certain urinary constituents as determined by changes in the character of the diet ... 161
Underhill, Frank P., and Baumann, Emil J. The interrelations of blood fat and blood sugar content of dogs under the influence of hydrazine ... 169

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FUNK, CASIMIR, LYLE, W. G., and McCASKEY, DONALD. The nutritive value of yeast, polished rice, and white bread, as determined by experiments on man........ 173
HOGAN, ALBERT G. The nutritive properties of corn........ 193
CSONKA, FRANK A. A colorimetric method for the determination of acetone in urine........ 209
WELLS, H. GIDEON, and HEDENBURG, O. F. The toxicity of carotin. 213
GEPHART, FRANK C., DU BOIS, EUGENE F., and LUSK, GRAHAM. Clinical calorimetry. XVIII. The number of places of significant figures in the data of metabolism experiments........ 217
HAAS, A. R. The permeability of living cells to acids and alkalies... 225
HAAS, A. R. The acidity of plant cells as shown by natural indicators. 233
TATUM, ARTHUR L. The influence of bile on autolysis........ 243
ADDIS, T., and WATANABE, C. K. The rate of urea excretion. II. The rate of excretion of administered urea in young healthy adults on a constant diet........ 249
ADDIS, T., and WATANABE, C. K. The volume of urine in young healthy adults on a constant diet........ 267
LUDEN, GEORGINE. Observations on the changes in the cholesterol content of the blood of goats, following cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment and subsequent castration........ 273
REED, GUILFORD B. The relation of oxidase reactions to changes in hydrogen ion concentration........ 299
FENGER, FREDERIC. Phosphatides in the ductless glands........ 303
LOEB, JACQUES, and NORTHROP, J. H. Nutrition and evolution. Second note........ 309
ECKLES, C. H., and PALMER, LEROY S. The influence of parturition on the composition and properties of the milk and milk fat of the cow........ 313
SCALES, F. M. A method for the determination of nitric nitrogen.... 327
LOEB, JACQUES. The mechanism of the diffusion of electrolytes through the membranes of living cells. I. The necessity of a general salt effect upon the membrane as a prerequisite for this diffusion........ 339
LOEB, JACQUES. The mechanism of the diffusion of electrolytes through the membranes of living cells. II. The diffusion of KCl out of the egg of *Fundulus* and the relative efficiency of different ions for the salt effect........ 353
LOEB, JACQUES. The mechanism of the diffusion of electrolytes through the membranes of living cells. III. The analogy of the mechanism of the diffusion for acids and potassium salts... 363
KURIYAMA, SHIGENOBU. The fate of alkali blue in the organism..... 377
ROBERTSON, T. BRAILSFORD. Experimental studies on growth. VIII. The influence of a diet deficient in fats, and of the same diet with cholesterol added, upon the growth of the white mouse.... 393

Salant, William, and **Bengis, Robert**. Physiological and pharmacological studies on coal tar colors. I. Experiments with fat-soluble dyes.......... 403
Robertson, T. Brailsford, and **Schmidt, Carl L. A.** An electrically heated vacuum desiccator.......... 429
Levene, P. A., and **Allen, C. H.** The oxidation of branched chain fatty acids. I. The action of hydrogen peroxide on the homologues of isobutyric acid.......... 433
Mueller, J. Howard. The mechanism of cholesterol absorption.... 463
Murlin, J. R., and **Kramer, B.** Pancreatic diabetes in the dog. I. The influence of alkali and acid upon the glycosuria and hyperglycemia.......... 481
Kramer, B., **Marker, J.**, and **Murlin, J. R.** Pancreatic diabetes in the dog. II. Is the glucose retained when sodium carbonate is administered to depancreatized dogs deposited as glycogen?.... 499
Murlin, J. R., and **Kramer, B.** Pancreatic diabetes in the dog. III. The influence of alkali on the respiratory metabolism after total and partial pancreatectomy.......... 517
Index to Vol. XXVII.......... 539

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Dean of the College of Agriculture and Director of the Agricultural
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Collaborator on the Journal of Biological Chemistry

Born . . . . . January 25, 1864
Died . . . . September 23, 1916

THE STUDY OF CERTAIN DIETARY CONDITIONS BEARING ON THE PROBLEM OF GROWTH IN RATS.

By CASIMIR FUNK.

In Cooperation with Joseph Poklop.

(*From the Huntington Fund, Memorial Hospital and Loomis Laboratory, and the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

(Received for publication, August 12, 1916.

During the progress of a series of investigations carried on by the writer in collaboration with Dr. Macallum,[1] it was deemed advisable to note in a series of control experiments the influence exerted by the addition of various specific substances, which might, not only from the standpoint of nutrition, but also from the standpoint of correcting deficiencies in diet, bring about a better condition of the experimental animals. The results obtained, covering a period of nearly 2 years and representing selected data noted by observation of several hundred rats experimented upon, are herewith presented.

As a matter of experience it might be stated at this time that the animals furnished to us by dealers seemed to lack that power of resistance which would make them suitable for our feeding experiments. This was manifested by a loss of hair on being placed in the metabolism cages, and early death before our experiments were well under way. This difficulty was promptly eliminated when rats bred in our laboratory under proper dietary conditions were substituted. The animals which were used for breeding purposes thrive best when kept on a diet of oats, bread, condensed milk, and yeast. The success of this diet was evidenced by the production of better animals from the physical, as well as from the experimental standpoint, than are produced by any other ways yet brought to our notice. Details of this dietary are shown elsewhere in the present paper.

---

[1] Funk, C., and Macallum, A. B., *J. Biol. Chem.*, 1916, xxvii, 51, 63.

In sequence to our earlier studies as to the value of oats as an exclusive diet for guinea pigs and rabbits,[2] our later experiments tend to show that oats, whether in seed or germinated, in conjunction with sodium bicarbonate or alone, were also an insufficient or unsuitable diet for young rats.

Our experiments show that the quantity of vitamines necessary for stimulating growth in rats is by no means small. If yeast is added to the diet to the extent of 1 per cent the rats grow for a short time, after which they begin to decline. Experiments which are not recorded in this paper have shown that at least 3 per cent of yeast is necessary to insure a satisfactory growth in rats. Still further experiments have shown that yeast has more effect in promoting growth than an addition of a few cc. of milk, as used by Hopkins.[3] Yeast can be regarded as a complete food by itself. It was therefore necessary to ascertain whether the good results obtained with this addition are not merely due to a correction of the nutritive value of the protein used (in this case, casein) or to the presence in it of nucleic acid. Consequently a diet was prepared in which the total casein nitrogen was substituted by yeast nitrogen. The results obtained were not as satisfactory as when yeast was used in smaller amounts for its vitamine content only, and not for nutritive value.

As orange juice has shown its value as a preventative of scurvy in rats, its value in promoting growth had to be ascertained. A diet which was made with orange juice instead of yeast was found to be without value for growing rats.

Finally an inquiry into the value of Lloyd's reagent as a precipitant for the growth-promoting substance in yeast was attempted. This reagent has been introduced by Seidell[4] for a quantitative separation of beri-beri vitamine from autolyzed yeast. He claimed that an addition of 50 gm. of the reagent to a liter of autolyzed yeast is sufficient for this purpose. Our results seem to show that a complete separation of the growth substance could not be effected, since both the precipitate and the filtrate were active, although to a lesser degree than the original solution, the activity of the precipitate being far more marked than that of the filtrate. We are not prepared to state at present whether our findings were due to the use of an insufficient amount of the reagent, or whether the separation of the growth-promoting factor into two fractions would be an explanation. We may be able to make a more definite statement in the near future. That the value of autolyzed yeast for growth experiments, as well as its antiscorbutic properties, for rats, is diminished by the above process, there is no doubt in our minds.[5]

---

[2] Funk, *J. Biol. Chem.*, 1916, xxv, 409.

[3] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

[4] Seidell, A., *U. S. Public Health Report*, No. 325, 1916.

EXPERIMENTAL.

Experiment 1. The Effect of Condensed Milk (Rats 3 and 4, Fig. 2).

The diet consisted of cabbage, oats, white bread, fresh vegetables, and condensed milk. The female became pregnant after 48 days and the experiment had to be discontinued. An unusual appetite for condensed milk seemed to be manifested by these animals which suggested to us that a possible increased supply of this form of milk might bring about still better results. The curves compare very favorably with the curves of control rats published by previous investigators and this suggests that careful description of the composition of normal diet is desirable.

Rats 3 (male) and 4 (female).

Days.	Weight. 3.	Weight. 4.
	gm.	*gm.*
0	41.8	35.0
4	60.0	48.7
8	66.9	55.4
12	83.4	68.3
16	95.6	75.9
20	108.7	84.2
24	122.5	93.2
28	134.8	99.4
32	142.5	104.4
36	149.7	116.2
40	146.7	121.4
44	148.7	131.7
48	158.6	154.5

---

[5] A short time ago a paper was published by my former assistant (Drummond, J. C., *Biochem. J.*, 1916, x, 77) in which he criticizes my results upon chickens stunted by a diet of unpolished rice. In answer I might state that, although his actual figures fully corroborate my statements, his main objection seems to be that even chickens on normal diet do not thrive when kept under laboratory conditions. This fact, of which I am

Experiment 2. The Effect of Yeast Addition to a Normal Diet (Rats 5 and 6, Fig. 2).

The diet consisted of cabbage, oats, white bread, fresh vegetables, condensed milk, and 2 gm. of dried yeast daily. The appearance of these rats as compared with those on artificial diets showed a better growth and a healthier general condition.

Rats 5 (male) and 6 (female).

Days.	Weight. 5.	Weight. 6.
	gm.	*gm.*
0	50.0	49.5
4	69.2	69.8
8	84.5	82.3
12	100.0	92.2
16	107.5	96.4
20	122.6	122.1
24	133.1	123.1
28	146.4	138.2
32	141.3	133.6
36	160.0	150.8
40	172.8	154.3
44	178.5	161.0
48	190.0	168.6
52	199.2	181.1
56	206.1	191.3
60	216.0	196.7
64	217.0	197.8

*Experiment 3. The Value for Growth and Maintenance of Oats Alone or with Sodium Bicarbonate.**

Rats 29, 30, and 31 received oats alone and Rats 32, 33, and 34 oats with 1 per cent sodium bicarbonate. All the animals showed practically no growth and died. The addition of the alkali has a distinct deleterious effect (Fig. 1).

---

fully aware, has been already emphasized in one of our publications. (Funk and Macallum, *Z. physiol. Chem.*, 1914, xcii, 13). Still it also remains a fact that as a result of an inquiry into conditions existing in poultry farms, we are using now a mixture of chicken food, cabbage, and charcoal, the water being entirely replaced by milk, thereby succeeding in diminishing the mortality to a very great extent, in spite of the fact that the birds are kept in small cages. With this improvement we have obtained new data confirming our previous statements on the effect of unpolished rice in arresting the growth of young chickens.

	Rats 29, 30, and 31. Oats alone. Weight (gm.).			Rats 32, 33, and 34. Oats and sodium bicarbonate. Weight (gm.).		
Days.	29.	30.	31.	32.	33.	34.
0	69.6	68.8	68.8	41.6	47.0	56.0
4	70.3	69.7	65.3	41.0	45.6	59.3
8	68.5	58.5	57.8	46.0	42.0	58.3
12	72.6	59.0	61.4	42.0	39.5	55.7
16	75.8	57.7	61.5	34.2		60.4
20	75.5	54.2	59.0			57.6
24	74.4	53.3	55.9			55.0
28	72.8	51.1	52.0			52.1
32	71.4	50.8	51.5			
36	73.6	50.7	52.0			
40	72.8	52.5	50.5			
44	68.5	54.0	44.4			
48	62.2	48.6				
52	63.0	47.0				

Experiment 4. Germinated Oats as Compared with Plain Oats in Their Value for Growth.

These experiments have been performed in analogy to the investigation of Fürst[6] who has found that germinated oats develop antiscorbutic properties (using guinea pigs). It was possible that rats would grow better on germinated seeds, with green parts already developed, but this was not to be the case and strangely enough the animals on oats alone outlived the others. The rats (21, 22, 23, and 24, Fig. 1) received at the beginning germinated oats, which were later supplemented with white bread. Rats 25, 26, 27, and 28 received plain oats and later also white bread. From the 18th day both lots had an artificial diet mixed with 5 per cent plain oats or 10 per cent germinated oats, the latter being found twice as heavy in our case.

---

[6] Fürst, *V.*, *Z. Hyg. u. Infectionskrankh.*, 1912, lxxii, 121.

	Rats 21, 22, 23, and 24. 0–3 days, germinated oats. 3–18 days, addition of white bread. From the 18th day, Diet 1.	Rats 25, 26, 27, and 28 0–3 days, plain oats. 3–18 days, addition of white bread. From the 18th day, Diet 2.
	Diet 1.	Diet 2.
	gm.	gm.
Casein	22	22
Sugar	10	10
Starch	23	28
Lard	30	30
Agar	2	2
Salts	3	3
Germinated oats	10	
Plain oats		5

Days.	Weight (gm.).				Weight (gm.).			
	21.	22.	23.	24.	25.	26.	27.	28.
0	39.5	58.0	44.5	33.1	40.3	35.6	36.9	51.0
4	41.3	64.0	48.6	37.3	45.1	40.2	39.7	53.5
8	42.0	51.0	48.2	36.8	44.4	39.8	42.2	54.8
12	39.6	62.2	46.8	34.2	44.8	38.8	41.4	54.6
16	39.4	69.0	49.5	35.0	46.0	40.5	46.3	54.9
20	31.7	56.2	46.5	33.1	48.0	39.5	52.3	56.3
24	31.7	56.2	46.5	33.1	51.9	41.2	53.5	54.3
28			33.4		54.3	37.0	53.2	53.8
32			37.5		54.3	40.4	44.0	51.8
36			37.0		61.4	50.2		56.0
40					65.6	54.4		56.1
44					70.2	58.0		56.0
48					72.0	61.0		55.8
52					72.8	62.2		51.5
56					66.0	55.5		
60					66.5	54.8		

Experiment 5. The Effect of an Addition of Milk to an Artificial Diet.

The animals received the usual artificial diet and in addition to that at the beginning of the experiment 1 cc., later 2 cc. of milk each. The growth of the animals was decidedly slower than on autolyzed yeast. The

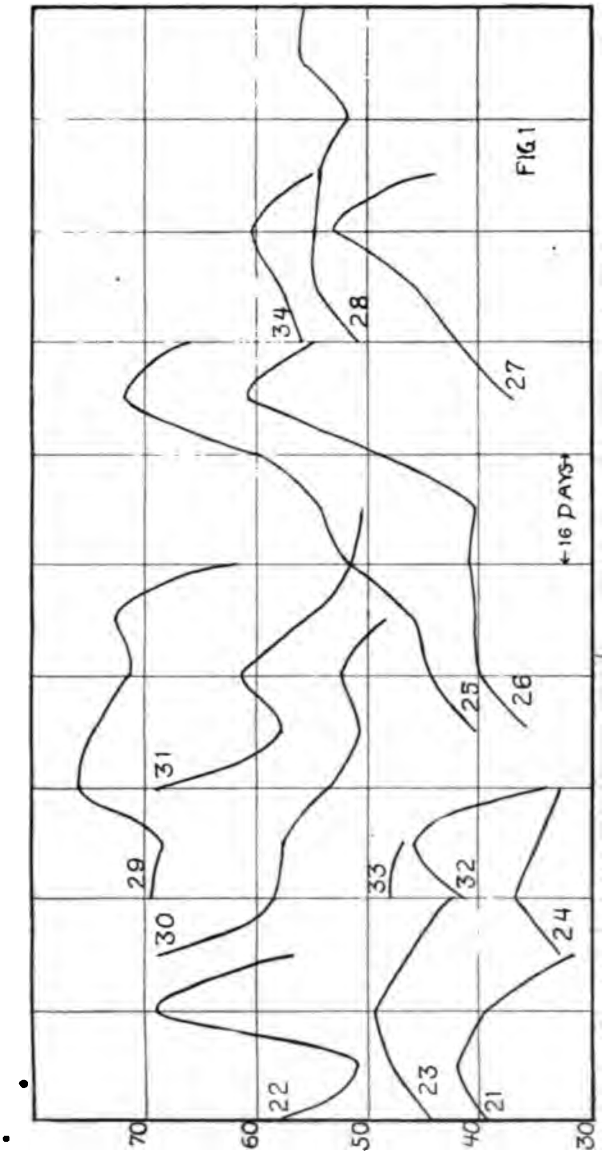

Fig. 1.

	Rats 21, 22, 23, and 24. 0–3 days, germinated oats. 3–18 days, addition of white bread. From the 18th day, Diet 1.				Rats 25, 26, 27, and 28 0–3 days, plain oats. 3–18 days, addition of white bread. From the 18th day, Diet 2.			
	Diet 1.				Diet 2.			
	gm.				*gm.*			
Casein	22				22			
Sugar	10				10			
Starch	23				28			
Lard	30				30			
Agar	2				2			
Salts	3				3			
Germinated oats	10							
Plain oats					5			
Days.	Weight (gm.).				Weight (gm.).			
	21.	22.	23.	24.	25.	26.	27.	28.
0	39.5	58.0	44.5	33.1	40.3	35.6	36.9	51.0
4	41.3	64.0	48.6	37.3	45.1	40.2	39.7	53.5
8	42.0	51.0	48.2	36.8	44.4	39.8	42.2	54.8
12	39.6	62.2	46.8	34.2	44.8	38.8	41.4	54.6
16	39.4	69.0	49.5	35.0	46.0	40.5	46.3	54.9
20	31.7	56.2	46.5	33.1	48.0	39.5	52.3	56.3
24	31.7	56.2	46.5	33.1	51.9	41.2	53.5	54.3
28			33.4		54.3	37.0	53.2	53.8
32			37.5		54.3	40.4	44.0	51.8
36			37.0		61.4	50.2		56.0
40					65.6	54.4		56.1
44					70.2	58.0		56.0
48					72.0	61.0		55.8
52					72.8	62.2		51.5
56					66.0	55.5		
60					66.5	54.8		

Experiment 5. The Effect of an Addition of Milk to an Artificial Diet.

The animals received the usual artificial diet and in addition to that at the beginning of the experiment 1 cc., later 2 cc. of milk each. The growth of the animals was decidedly slower than on autolyzed yeast. The

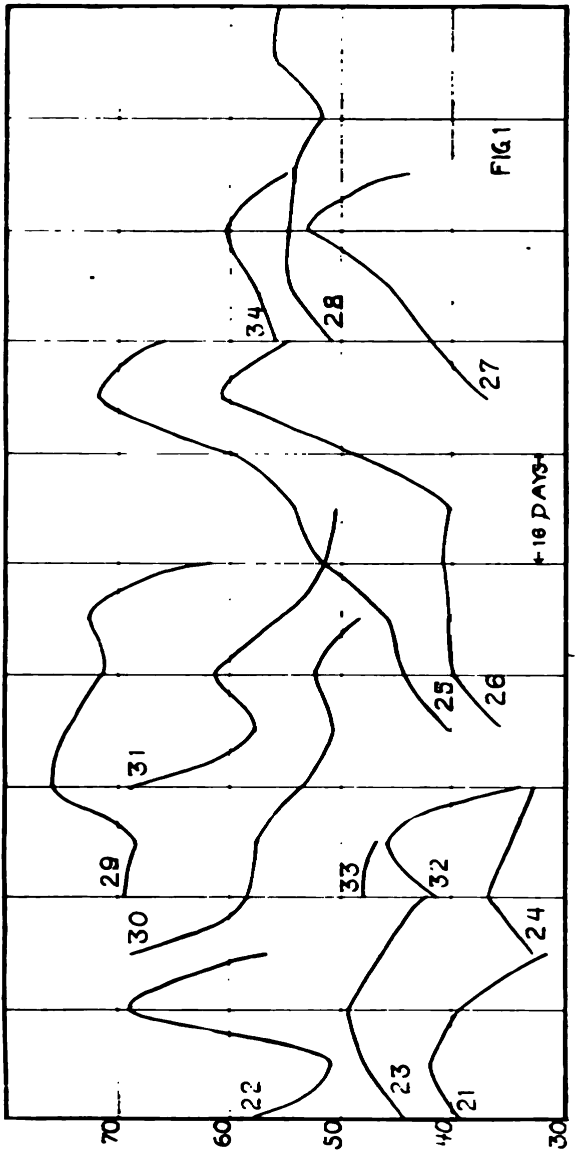

Fig. 1.

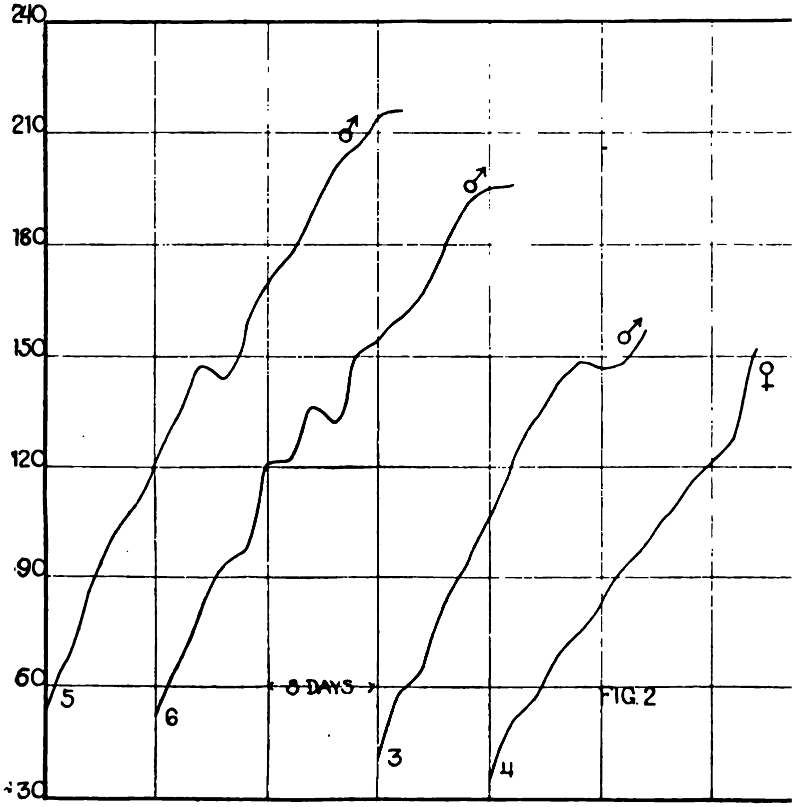

FIG. 2.

table below and the curves for Rats 35 (male) and 36 (female) are represented in Fig. 4.

	gm.
Casein	22
Sugar	10
Starch	33
Lard	30
Agar	2
Salts	3

0–20 days 1 cc. of milk.
20–56 " 2 " " "

Days.	35. Male. Weight.	36. Female. Weight.	Food intake.
	gm.	gm.	gm.
0	33.7	36.6	
4	39.5	41.4	8.5
8	44.1	45.6	7.6
12	49.4	47.4	8.7
16	53.2	47.7	6.3
20	53.7	46.3	6.2
24	52.0	44.8	5.9
28	52.6	45.6	5.6
32	51.5	44.7	5.5
36	59.7	47.8	5.5
40	64.8	47.2	7.5
44	64.0	48.0	5.1
48	63.9	48.7	5.2
52	63.3	51.4	4.4
56	66.0	52.6	3.6

Experiment 6. The Value of Yeast as Protein Supplier.

Casein in our artificial diet was substituted by the same quantity of nitrogen in form of yeast. One male (37) and one female (38) are recorded in this experiment. Rat 37 died with edema after 44 days. As the food intake came gradually down yeast cannot be regarded as a good substitute for protein. There is even a slight indication of a toxic action. Rat 38 which survived 52 days of this experiment has shown an inhibited growth in the last period (Fig. 3).

	gm.		gm.
Yeast	36	Lard	30
Sugar	10	Agar	2
Starch	20	Salts	2

Days.	Weight.		Food intake.
	37. Male.	38. Female.	
	gm.	*gm.*	*gm.*
0	33.3	39.0	
4	45.4	52.3	9.1
8	45.7	65.4	9.8
12	50.3	70.1	10.8
16	56.3	79.2	10.5
20	61.8	86.0	12.4
24	60.1	89.2	11.4
28	58.7	92.2	10.1
32	58.0	97.4	10.5
36	64.0	99.1	10.7
40	65.0	102.0	10.5
44	61.1	103.8	9.8
48		107.6	6.7
52		108.4	5.6

Experiment 7. Action of Orange Juice on Growth.

Rats 53 and 54, males (Fig. 4), were kept on an artificial diet in which the yeast was replaced by orange juice with the result that growth was completely arrested. The animals died after 5 weeks.

	gm.		*gm.*
Casein	22	Agar	2
Sugar	10	Salts	3
Starch	23	Orange juice	10
Lard	30		

Days.	Weight.		Food intake.
	53.	54.	
	gm.	*gm.*	*gm.*
0	28.5	30.0	
4	35.0	35.5	6.6
8	36.0	38.5	5.7
12	37.5	38.5	5.1
16	36.5	37.5	5.6
20	37.5	39.0	5.3
24	38.5	40.0	4.9
28	35.0	36.0	4.6
32	36.0	34.0	4.4
36	31.5		3.0

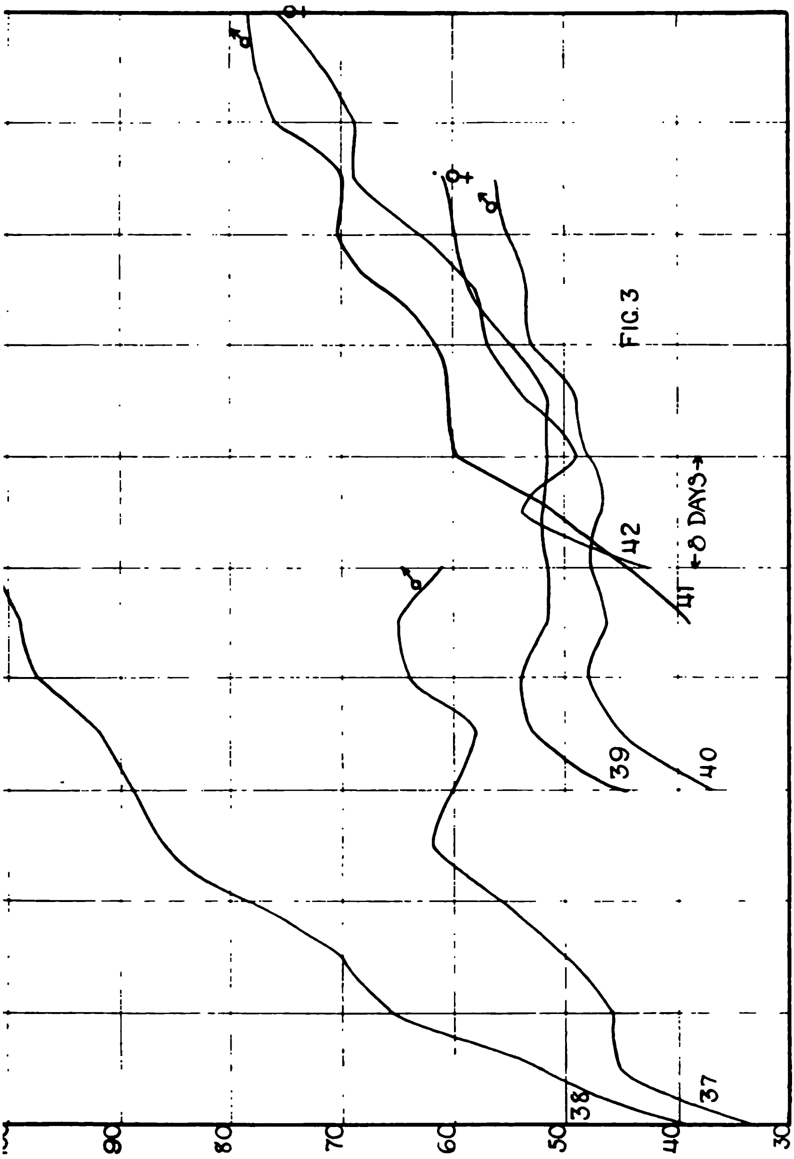

Fig. 3.

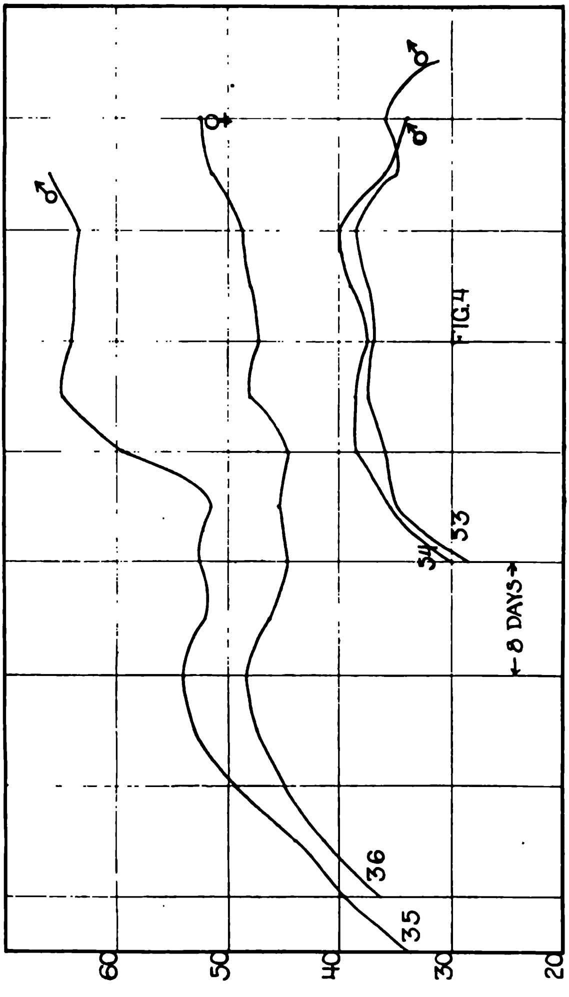

Fig. 4.

Experiment 8. The Value of Lloyd's Reagent as Precipitating Agent for the Growth-Promoting Substance in Yeast.

The precipitation with this reagent was made according to the method used by Seidell. The quantity of the precipitate and the filtrate added to the diet corresponded to 10 cc. of autolyzed yeast which gave good results in our hands. Rats 39 (female) and 40 (male) were kept on the precipitate for 44 days and Rats 41 (male) and 42 (female) (Fig. 3) for the same time on the filtrate. The animals kept on Lloyd's reagent grew decidedly better than those on the filtrate, but the separation of the growth-promoting substance was incomplete. Autolyzed yeast has lost, apparently, most of its antiscorbutic properties by this process.

	Diet 1.	Diet 2.
	gm.	*gm.*
Casein	22	22
Sugar	10	10
Starch	24	24
Lard	30	30
Agar	2	2
Salts	2	2
Lloyd's precipitate made up to	10 cc.	
Lloyd's filtrate		10 cc.

Rats 39 and 40, Diet 2. Rats 41 and 42, Diet 1.

Days.	Weight.		Food intake.	Weight.		Food intake.
	39. Female.	40. Male.		41. Male.	42. Female.	
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	44.9	37.0		39.1	43.4	
4	52.8	45.4	9.8	49.5	54.0	11.2
8	54.1	48.0	7.5	55.8	43.7	8.6
12	51.6	46.7	5.8	59.7	51.7	7.9
16	51.6	47.7	7.0	60.5	57.4	8.9
20	51.9	46.9	6.0	61.6	58.0	8.8
24	51.6	47.9	5.5	66.5	63.2	6.3
28	53.6	49.4	6.2	70.2	69.1	9.5
32	55.0	52.5	6.6	70.0	69.0	9.5
36	58.8	52.9	6.2	76.0	72.7	7.5
40	60.0	55.2	5.5	77.8	76.0	9.3
44	61.0	56.3	5.1	78.5	81.2	7.9

SUMMARY.

By an addition of milk or yeast to the normal diet the growth of rats can be accelerated. Oats in dry state or subjected to germination proved to be an inadequate diet for young rats. Yeast cannot substitute the casein in the diet and this very likely because of its toxic properties. Young rats grow less when milk instead of yeast is used in the diet and they do not grow at all on orange juice as addition. Using Lloyd's reagent for precipitation of the growth-promoting substance from autolyzed yeast, it was found that by this process the separation seems to be not complete; furthermore, the yeast loses some of its original value as a stimulant to growth.

COTTONSEED MEAL AS AN INCOMPLETE FOOD.

BY C. A. WELLS AND P. V. EWING.

(*From the Georgia Experiment Station, Experiment.*)

(Received for publication, July 11, 1916.)

Within the past few years it has been well established that certain injuries to animals, such as beri-beri, result from deficient diets. Thus, purified proteins, carbohydrates, fats, and mineral matter, combined in any proportion, do not produce normal growth. A ration made up of whole wheat, or of certain other cereals, will produce injury through lack of essential food substances. A diet is incomplete if it does not contain an adequate amount of these as yet unidentified accessory substances as well as requisite inorganic salts.

Rommel and Vedder (7) recently advanced the hypothesis, supported by preliminary experiments, that cottonseed meal injury is similar to, if not indeed identical with beri-beri in man. Our own experiments (8) had indicated that in feeding cottonseed meal to pigs to ascertain the injury said to result from this it is necessary to balance the ration, not so much as to the nutritive ratio, determined by the amounts of fats, carbohydrates, and protein present, but rather as to the so called accessory food factors. It is the purpose of the present paper to relate our later experiments bearing upon this phase of the cottonseed meal injury question.

EXPERIMENTAL.

Equipment.—Mated Duroc-Jersey pigs were used. These were 6 weeks old and weighed usually about 6 to 10 kg. each at the beginning of the experiment. They were kept in pens or metabolism cages and fed under conditions previously described in detail (8). We used the pig because it is said to be more susceptible to cottonseed meal injury than are other animals, thus making it easier, presumably, to measure the injury; and when results are obtained with it there is not the further implied necessity of comparative studies as is always the case where rabbits and similar animals are used. It is inexpensive, and lends itself satisfactorily to metabolism trials in cages.

Feeds.—The feedstuffs were obtained, analyzed, and kept in dry storage throughout the investigation. From the analyses of them, and from the energy values of feeds as given by Armsby and others, the dry matter, digestible protein, and net energy values were calculated on the basis of the feed consumed. The cottonseed meal was of a good grade having a bright yellow color and a sweet odor. The digester tankage was a commercial grade, and the gluten was a good grade of Hoyt's gluten flour. The whole milk was obtained from a Jersey herd and modified by mixing two parts of whole milk with one part of skimmed milk. Preliminary feeding trials were made in each case with a mixture of corn meal 62, wheat middlings 30, and digester tankage 8 parts.

TABLE I.

Dry Matter and Digestible Protein, and Net Energy Values in Therms in 100 Gm. of Air-Dried Substance.

Substance.	Total dry matter.	Nitrogen.	Digestible protein.	Net energy value.
	gm.	*gm.*	*gm.*	*therms*
Corn meal	89.1	1.50	8.4	0.180
Wheat middlings	84.0	2.80	12.8	0.171
Digester tankage	92.7	8.46	47.1	0.161*
Cottonseed meal	91.5	6.66	55.6	0.186
Starch	87.3			0.223
Sugar	100.0			0.256*
Skimmed milk	9.9	0.50	3.6	0.019
Whole (diluted) milk	8.7	0.48	3.4	0.024
Gluten flour	91.3	12.03	63.8	0.180

* Energy values calculated.

In Series 1 a study was made of the comparative effects of large quantities of cottonseed meal, digester tankage, and gluten flour in a limited basal ration; and similarly in Series 2, using cottonseed meal and gluten flour in a basal ration increased to absolute maintenance. In the latter series metabolism trials were made. The results are summarized in Tables II, III, and IV.

TABLE II.

Series 1. Effects of Large Quantities of Cottonseed Meal, Digester Tankage, and Gluten Flour in a Limited Basal Diet. Daily Averages in Periods.

Date.	Weight.	Feed consumed.	Cottonseed meal.	Additional water.	Dry matter.	Digestible protein	Net energy value.	Injury.	Death.
Pig 1. Diet: Cottonseed meal 25 gm., starch 6, sugar 2.3, skimmed milk 5 cc., per kg. live weight daily.									
1916	*kg.*	*gm.*	*gm.*	*cc.*	*gm*	*gm.*	*therms*		
July 1-10....	10.0	383	250	1,223	305	101	0.64	−	−
" 10-20....	10.5	296	203	1,088	248	82	0.52	+	−
" 20-30....	10.0	223	163	904	200	66	0.42	+	−
Aug. 1-10....	10.0	201	151	1,138	184	61	0.38	+	−
" 10-18....	10.0	148	104	1,050	127	42	0.27	+	−*
Pig 2. Diet: As for Pig 1, with 1.5 gm. gluten flour instead of 5 cc. skimmed milk.									
July 1-10....	10.0	383	250	1,137	314	99	0.66	−	−
" 10-20....	10.0	369	245	1,134	308	97	0.64	+	−
" 20-30....	10.0	333	221	1,116	278	87	0.58	+	−
Aug. 1-10....	11.0	275	190	868	236	75	0.50	+	−
" 10-15....	10.0	120	83	610	104	32	0.22	+	+
Pig 3. Diet: As for Pig 1, with 2.5 gm. digester tankage instead of 5 cc. skimmed milk.									
July 1-10....	10.0	383	250	1,110	320	102	0.67	−	−
" 10-20....	11.0	421	265	1,310	339	108	0.71	+	−
" 20-30....	12.0	311	186	964	238	76	0.50	+	−
Aug. 1- 8....	14.0	224	145	738	185	59	0.40	+	+
Pig 4. Diet: As for Pig 1, with 12.5 gm. gluten flour instead of 25 gm. cottonseed meal.									
July 1-10....	10.0	258		1,304	190	92	0.40	−	−
" 10-20....	10.5	253		1,030	154	75	0.30	+	−
" 20-30....	10.0	202		865	124	60	0.25	+	−
Aug. 1-10....	10.0	187		1,202	115	56	0.24	+	−
" 10-18....	10.0	129		951	79	38	0.17	+	+

* Removed at close of last period after complete injury, to prevent death.

TABLE II—*Concluded.*

Pig 5. Diet: As for Pig 1, with 18.5 gm. digester tankage instead of 25. gm. cottonseed meal.

Date.	Weight.	Feed consumed.	Cottonseed meal.	Additional water.	Dry matter.	Digestible protein.	Net energy value.	Injury.	Death.
1915	*kg.*	*gm.*	*gm.*	*cc.*	*gm.*	*gm.*	*therms*		
July 1–20....	10.0	268		1,252	245	87	0.47	–	–
" 20–Aug. 10....	12.0	294		1,060	267	94	0.56	+	–
Aug. 10–30...	13.0	232		985	210	74	0.45	+	–
" 31–Sept. 20....	13.0	235		983	212	75	0.46	+	–
Sept. 20–Oct. 9......	13.0	211		1,114	192	69	0.42	+	–
Oct. 9–23....	10.0	58		389	53	19	0.12	+	+

DISCUSSION.

It is evident that submaintenance and failure to grow were not due to lack of supply of carbohydrates, fats, protein, and mineral matter. The diets were deficient in some other essential way; so that the value of a diet is not to be measured solely by the amount of protein and net energy it contains.

The lethal period was longer than that previously reported by us (8). This was due, no doubt, to the presence here of additional substances in the diet, such as the small quantity of milk. Dinwiddie (1) found much the same thing in that wheat bran seemed to lessen the injury from cottonseed meal, and the reports of others show that animals do not suffer so much from cottonseed meal if given a variety of food or access to pasture.

Apparently the digester tankage was a more complete food than cottonseed meal or gluten flour. It must have yielded, relatively, a larger proportion of the less abundant valuable food factors, such as tryptophane. Or, in the case of Pigs 1, 2, and 3, the diets may have contained a toxic substance.

The injury was not acute. Through physical appearances and the metabolic characteristics it was manifested long before death. This is not in agreement with the observations of many

TABLE III

Series 2. Effects of Cottonseed Meal and Gluten Flour when Fed in Low, Medium, and High Maintenance Diets. Daily Averages by Periods.

Date.	Diet.	Feed consumed.	Additional milk.	Additional water.	Total feed consumed.			Weight.	Gain or loss in weight.
					Dry matter.	Digestible protein.	Net energy.		
Pig 6. Experimental diet: Cottonseed meal 3 gm., starch 6, sugar 2.3, per kg. live weight daily, with whole milk as indicated.									
1915-16		*gm.*	*cc.*	*cc.*	*gm.*	*gm.*	*therms*	*kg.*	*gm.*
Dec. 20–23	Preliminary.	236		1,210	203	30.0	0.40	7.5– 7.9	+100
" 24–Jan. 10	Experimental.	80	164	540	82	8.6	0.19	8.2– 6.9	−117
" 11–22	"	80	213	581	83	9.2	0.20	6.1– 6.7	+ 50
" 23–Mar. 20	"	96	630	1,883	86	10.4	0.21	6.7–13.3	+150
Mar. 21–May 5	Recuperative.	720	1,500		769	141.0	1.52	13.3–30.0	+458
Pig 7. Experimental diet: As for Pig 6, with 2.36 gm. digester tankage instead of 3 gm. cottonseed meal.									
Dec. 20–23	Preliminary.	210		1,356	181	27.0	0.36	6.7– 7.2	+120
" 24–Jan. 10	Experimental.	67	126	688	69	6.0	0.16	7.3– 5.5	− 95
Jan. 11–22	"	67	193	856	70	6.5	0.17	5.5– 5.7	+ 17
" 23–Mar. 20	"	78	588	2,166	84	8.5	0.21	5.7–11.2	+ 96
Mar. 21–May 5	Recuperative.	653	1,370		700	122.0	1.38	11.2–28.0	+410
Pig 8. Experimental diet: As for Pig 6, with 1.66 gm. gluten flour instead of 3 gm. cottonseed meal.									
Dec. 20–23	Preliminary.	223		956	192	28.0	0.38	7.1– 7.7	+150
" 24–Jan. 10	Experimental.	67	154	435	69	6.0	0.16	7.7– 6.0	− 94
Jan. 11–22	"	67	210	343	70	6.5	0.17	6.0– 6.2	+ 17
" 23–Mar. 20	"	78	637	1,550	84	8.5	0.21	6.2–12.0	+102
Mar. 21–May 5	Recuperative.	590	1,240		627	112.0	1.25	12.2–30.0	+434

TABLE III—*Concluded.*

Date.	Diet.	Feed consumed.	Additional milk.	Additional water.	Total feed consumed.			Weight.	Gain or loss in weight.
					Dry matter.	Digestible protein.	Net energy.		
Pig 9. Experimental diet: As for Pig 6, with 22.5 gm. cottonseed meal instead of 3 gm. of cottonseed meal. Milk fed separately.									
1915–16		*gm.*	*cc.*	*cc.*	*gm.*	*gm.*	*therms*	*kg.*	*gm.*
Dec. 20–23	Preliminary.	213		493	183	27.0	0.36	6.8–7.2	+100
" 24–Jan. 10	Experimental.	150	144	1,047	137	40.0	0.31	7.2–6.0	− 33
Jan. 11–22	"	120	210	357	111	33.0	0.25	6.0–5.6	− 33
" 23–25	"	54	392	304	55	16.0	0.13	5.6–5.5	− 33
Pig 10. Experimental diet: As for Pig 6, with 12.45 gm. gluten flour instead of 3 gm. cottonseed meal. Milk fed separately.									
Dec. 20–23	Preliminary.	200		1,050	172	26.0	0.34	6.2–6.9	+125
" 24–Jan. 10	Experimental.	125	134	653	114	33.0	0.25	6.9–5.9	− 55
Jan. 11–12	"	125	206	730	115	34.0	0.26	5.9–6.9	− 83
" 13–24	"	25	483	580	29	10.0	0.07	6.9–5.5	−120
Pig 11. Experimental diet: As for Pig 9, with 70 cc. whole milk per kg. live weight daily, fed separately from beginning of experimental period.									
Jan. 22–25	Preliminary.	330		600	284	42.0	0.56	9.9–10.0	+ 25
" 26–Apr. 26	Experimental.	105	875	865	102	30.0	0.23	10.0–15.1	+ 55
Apr. 27–May 11	Recuperative.	503	1,057		536	137.0	1.05	15.1–25.5	+700
Pig 12. Experimental diet As for Pig 11, with 12.4 gm. gluten flour instead of 22.5 gm. cottonseed meal.									

Date.	Pig.	Weight.	Diet.	Volume.	Acidity. Log.	Ammonia nitrogen.	Urea nitrogen.	Total nitrogen.	Creatinine.	Sulfur oxidised.	Total sulfur.	Weight.	Nitrogen.	Nitrogen balance.	Nitrogen utilization.
1915–16		kg.		cc		gm.	gm.	gm.	gm.			gm.	gm.		per cent
Dec. 20–23..	6	7.1	Preliminary, 236 gm.	154	7.4	0.284	1.161	1.804	0.184	0.101	0.140	49	1.112	+2.748	80
Apr. 18–20..	6	17.0	Recuperative, 567 gm. with 1,200 cc. milk.	963	7.0	2.141	2.874	6.631	0.753	0.438	0.575	567	7.964	+5.113	76
Dec. 20–23..	9	6.4	Preliminary, 213 gm.	132	6.2	0.102	0.588	1.365	0.173	0.075	0.115	65	1.248	+2.499	76
Jan. 10–12..	9	7.2	Experimental. Ate 144 gm. cottonseed meal with 144 cc. milk.	225	7.2	0.126	2.059	2.174	0.071	0.107	0.136	57	1.532	+6.504	85
Dec. 20–23..	10	6.0	Preliminary, 200 gm.	195	7.1	0.281	1.152	1.710	0.220	0.134	0.185	56	1.220	+1.870	76
Jan. 10–12..	10	7.7	Experimental. Ate 95 gm. gluten flour with 138 cc. milk.	330	6.9	0.504	6.237	6.930	0.116	0.285	0.343	12	0.466	+4.694	96
Dec. 20–23..	11	6.3	Preliminary, 210 gm.	245	7.4	0.240	1.132	1.739	0.178	0.112	0.139	108	1.248	+2.125	75
Apr. 13–15..	11	13.6	Experimental. Ate 124 gm. cottonseed meal with 952 cc. milk.	1,289	7.2	1.348	7.368	13.529	0.299	0.508	0.845	61	1.265	−1.776	90
May 9–11..	11	25.5	Recuperative, 850 gm.	1,330	7.0	2.088	4.223	8.472	0.891			485	7.265	+5.663	64
Dec. 20–23..	12	6.6	Preliminary, 220 gm.	185	6.5	0.320	1.147	1.584	0.170	0.189	0.231	92	1.180	+2.392	76
Apr. 13–15..	12	25.0	Experimental. Ate 311 gm. gluten flour with 1,750 cc. milk.	4,600	5.7	2.087	21.771	34.650	0.263	1.482	2.382	13	0.663	+10.819	98

other investigators. We are inclined to believe, however, that in the reported cases of acute cottonseed meal injury, careful periodic measurements would have disclosed the injury before death. This is important; for if well nourished animals die of the injury as often claimed, the same cannot be due to a deficient diet and inanition, but must be due to a toxic effect.

The ingestion of smaller quantities of nitrogenous foods as the injury advanced, and the retarding effect of additional nitrogenous foods, indicate that excess of nitrogen did not cause the injury. When upon an assured maintenance diet the sugar, starch, and other foods did not produce injury. And since the pigs, upon full maintenance, would not eat much cottonseed meal, it was necessary to feed the basal diet separately that it might all be eaten.

Postmortem examinations were made in all cases, but the results are omitted here, because, as shown in detail in a previous publication (8), they varied so widely as to have little interpretive value. Among those characteristics which were found most constant may be mentioned: a rough coat; dry, harsh, and scaly skin; weakness and emaciation as shown by physical appearance and entire absence of fat from kidneys and mesentery; and bones usually though not always brittle.

From results in Series 2 it appears that injury was not prevented by that amount of milk which Hopkins (3) found sufficient for protection of rats receiving a deficient diet. Even Dietrich's maintenance allowance for pigs was inadequate here. But *absolute maintenance was assured when the milk was increased to 70 cc. per kg. live weight daily.*

The death of Pig 10 was due probably to a tumor-like internal injury revealed by postmortem examination. In all other cases death resulted in part from lack of sustenance. It should be noted, however, that Pig 9 died 4 days after its maintenance was made absolute by the addition of milk. The fact that it did not respond, as did Pigs 6, 7, and 8, to this increased milk allowance, leaves the possibility to be reckoned with that the cottonseed meal contained a specific injurious effect apart from its lack of essential food factors.

The metabolism trials confirm the results formerly reported (8) to the effect that on such diets the pig is unable to deflect nitrogen from urea to form ammonia to neutralize any excess acids present. The low utilization of nitrogen in diets highly efficient for growth and its high utilization in diets inefficient for growth may be explained on the basis that the animals when on restricted diets utilized the nitrogen to the greatest possible extent in an effort to obtain those particular substances much needed yet present to a limited extent in the diet. This explanation, of course, is not in keeping with the commonly accepted view that the character of a ration does not affect the degree of utilization of any food constituent. Yet it should be borne in mind that Mendel and Fine (4, 5, 6) have shown that the proteins of corn, wheat, and meat are utilized in carnivora to approximately the same extent; namely, ± 93 per cent. One would expect, therefore, upon mixing these substances together, as was essentially the case in our preliminary and recuperative diets, to obtain an equivalent utilization of the nitrogen. But this did not happen. The nitrogen was utilized to approximately 76 per cent, which is about the figure given in the literature for this food mixture. Constant watch was kept to see that the pigs did not eat the feces; and from the results in Table IV it is evident that in the restricted diets anabolism was at its maximum, while in the case of the normal preliminary and recuperative diets much of the nitrogen went to waste. When on a bountiful diet, the animal apparently selected with ease those substances demanded by the specifically established physiological processes of the body, including nitrogen, letting much go to waste through the feces; but when the diet was restricted, the tendency was to increase the nitrogen utilization beyond that required by the body in an effort to obtain the other necessary substances. A reference to our related work on the associative action of foods with steers (2) where cottonseed meal was fed alone as a restricted diet, and with corn silage making a fairly normal diet, shows this same increased utilization of nitrogen on the restricted diet. The results, of course, are based upon limited data and are indicative rather than conclusive.

The decrease in creatinine in the urine when the pigs were changed from the normal to the experimental diets was not associated with the possibility of preformed creatinine in the digester tankage fraction, as shown by separate trials not recorded here. The creatinine decrease indicated a condition of inanition and impaired muscular activity. The creatinine not eliminated was utilized, perhaps, as food, for which there was an increased demand in the one-sided diets. Throughout it is evident that inanition played an important part in the injury.

The results show that in studies of so called cottonseed meal injury with pigs it is necessary to eliminate the interference of inanition by feeding a basal diet which contains those substances requisite for normal growth in addition to the dry matter, digestible protein, and net energy usually considered. The diet must contain milk or some combination of other foods of known growth-producing value.

CONCLUSIONS.

1. Cottonseed meal is an incomplete food. This is true even when it is fed with sugar and starch to a wide nutritive ratio.
2. Pigs upon an absolute maintenance diet ate in addition only small quantities of cottonseed meal and were not greatly injured by it.
3. So called cottonseed meal injury is due in large part to inadequate diets.

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THE PHYSIOLOGICAL RELATION OF PLANT CAROTINOIDS TO THE CAROTINOIDS OF THE COW, HORSE, SHEEP, GOAT, PIG, AND HEN.

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(From the Dairy Chemistry Laboratory, University of Missouri, Columbia.)

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It has been shown by the author[1] that the natural yellow pigments which characterize the milk fat and tissue fat of the cow, the milk fat of the human, and the egg yolk and tissue fat of the hen, owe their origin to the carotin and xanthophyll pigments present in the food. These natural plant pigments, which are collectively known as carotinoids, appear to be transferred in some simple fashion by the blood serum from the digestive tract to the fat-forming organs where they became incorporated with the synthesized fat. Just how this is brought about is at present obscure. The author has shown conclusively, however; that the presence of pigment in deposited tissue fat, or in the egg yolk, or in the milk fat depends exclusively upon the presence of the same pigment in the blood serum. For example, when cows were fed rations nearly devoid of carotinoids, these pigments practically disappeared from the blood serum, and the milk fat became almost colorless. Similarly, when laying hens were fed rations nearly devoid of carotinoids the blood serum, as well as the egg yolks, showed almost complete absence of these pigments.

Probably the most interesting result of the study of the yellow pigments which characterize animal fat has been the demonstration of the fact that the yellow pigments which are resorbed from the digestive tract of the cow consist almost exclusively of carotin, very small portions of the xanthophyll group of pigments being present. The pigment which characterizes the egg yolk and tissue fat of the hen, on the other hand, consists almost exclusively of xanthophyll, relatively little carotin being resorbed. The pigments of human milk fat, in the case of one woman studied by the author, appear to consist of much more nearly equal parts of the two classes of carotinoids.

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[1] Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191. Palmer, L. S., *ibid.*, 1915, xxiii, 261.

Another interesting fact developed with reference to the manner in which the blood serum of the cow and hen carries the pigment characteristic of the species. In the case of the cow the carotin of the blood serum is in some sort of physical or chemical combination with albumin. This compound, which the author has called caroto-albumin, is easily isolated from fresh serum. It is readily soluble in water, while carotin itself is soluble only in the fat solvents. In the case of the hen serum, however, no such combination appears to exist between xanthophyll and protein, the pigment being readily extracted from the serum with ether, which is in marked contrast to the complete failure of pure ether to extract any carotin from the fresh blood serum of the cow.

Carotinoids in Other Species of Animals.

The presence of carotin in the cow and xanthophyll in the hen, in each case to the relative exclusion of the other members of the carotinoid group, raised the question of what pigments characterize the tissue fat and blood serum of animals of other species. The author has recently had an opportunity to investigate this question in the case of the horse, pig, sheep, and goat. It is the purpose of this paper to set forth briefly the results of that study.

Carotinoids in Swine.—Considerable interest was attached to the study in the case of the swine, and also in the case of the sheep, reported below, on account of the fact that the tissue fat of each of these species is characteristically colorless. The question involved was whether this would be found to be due to differences between these species and the cow and hen at the seat of the fat synthesis, or to the failure of the carotinoids to be resorbed from the digestive tract.

Blood was secured from three pure-bred swine, representing the Duroc-Jersey, Poland China, and Berkshire breeds. For several weeks before they were slaughtered the animals had run on pasture containing an abundance of fresh blue-grass. The conditions were thus most favorable for the largest transfer of carotinoids into the blood stream. The blood from each of the animals was defibrinated at once, and the plasma centrifugalized. The serum was tested for the presence of carotin and xanthophyll by several methods. The reader is referred to the previous work by the author[2] for a description of these methods. Desiccation of the serum with plaster of Paris and extraction with petroleum ether and ether after moistening with absolute alcohol is the easiest and most reliable method for extracting the carotinoids from blood serum.

The results in the case of each of the three pigs were absolutely negative. Not a trace of either carotin or xanthophyll was present in the blood serum.

Carotinoids in Sheep.—Blood was secured from five pure-bred ewes, representing the following breeds: Dorset, Hampshire, Merino, Shropshire, and Southdown. Each of the ewes was in milk with the exception of the Hampshire. The animals received an abundance of fresh blue-grass pasture for several weeks before they were bled. The blood was secured from the jugular vein by means of a trocar, about 150 cc. being obtained from each animal. The four ewes in milk were milked at the same time the blood was drawn, the milk from the Dorset and Southdown ewes being combined, as was also that from the Merino and Shropshire ewes.

The blood from each animal was defibrinated, the plasma centrifugalized, and the serum examined for the presence of carotinoids. The extracts from 10 cc. portions of the serum failed to reveal any pigment whatever, but the combined extracts from about 150 cc. of serum, when concentrated to a volume of 5 to 10 cc. showed a pale yellow color, which was completely extracted from 85 per cent alcohol by both petroleum ether and carbon disulfide, indicating that the pigment was carotin. The merest traces of pigment thus appeared to be present in the blood of the five sheep examined.

The milk obtained from the ewes was churned, without separating, on account of the small amount obtained. The butter in each case was "dead" white in color, but when rendered it had a faint greenish tint which may have been due to the presence of carotin. Not enough butter fat was secured to investigate this point further.

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[2] Palmer and Eckles, *J. Biol. Chem.*, 1914, xvii, 223–236.

Carotinoids in the Goat.—Blood was secured from a she-goat of the Angora breed while receiving an abundance of fresh green feed. The goat was dry at the time, so that no milk was obtained.

The study of the blood serum indicated the possible presence of faint traces of carotin. The extract of 10 cc. of serum would have been judged colorless, however, had it not been compared carefully with a similar amount of fresh petroleum ether.

The study of the yellow pigmentation in the case of the goat was of special interest owing to the investigation of the transference of pigments into milk by Dombrowsky,[3] who fed a goat on carrots and observed whether the carotin passed into the milk. Two feeding trials 2 weeks apart resulted in the observation by Dombrowsky that the milk was yellowish on the evening of the 2nd day of the first feeding trial and was clearly yellowish on the 3rd and 4th days of the second trial. An attempt to show that the color was due to carotin resulted in failure in each case. The test used was the application of the well known color reaction of carotin with concentrated H_2SO_4, to the residue from an ether extract of the milk. If considerable carotin was present, which the author doubts, the test used by Dombrowsky would have been vitiated by the fat present in the extract, since no attempt was made to eliminate it. It is unfortunate that Dombrowsky failed to observe whether the fat in the milk showed an increased color accompanying the carrot feeding. A statement of the breed of the goat used would also have enhanced the value of his results.

Carotinoids in the Horse.—Blood serum was secured in the usual way from a horse which had been running on fresh bluegrass pasture for several weeks. The serum was found to be rich in carotin. No xanthophyll was found to be present. The manner in which the horse serum carries the carotin was found to be identical with that of the cow. The isolation of the water-soluble caroto-albumin from 100 cc. of serum was carried out with the greatest ease. The method used may be of interest to the reader and will be described briefly.

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[3] Dombrowsky, *Arch. Hyg.*, 1904, l, 183.

100 cc. of the fresh serum were diluted with an equal volume of water and treated with 200 cc. of saturated $(NH_4)_2SO_4$ solution. After standing 1 hour the globulin was filtered off on a Buchner, using suction. The deep yellow filtrate was heated carefully in a water bath to 79°C. The heavy coagulum which formed was filtered off on a Buchner, using suction. The golden yellow filtrate was treated with $(NH_4)_2SO_4$ in substance until precipitation of the pigmented protein occurred. This was filtered off, using suction, which was continued until the precipitate on the funnel was free from visible moisture. It had a dark yellowish brown appearance. It dissolved readily in 50 cc. of warm water, giving a deep yellow solution. It was not possible to extract the carotin from this solution until the pigment was first freed from the protein with alcohol.

The amount of carotin carried by a unit volume of horse serum was found to be considerably less than that carried by the serum of the cow. Where 5 cc. of horse serum gave an extract giving a reading of 3.75 units of yellow and 0.4 units of red in the Lovibond tintometer in a 1 inch layer and 12.5 cc. volume, a similar amount of cow serum under like conditions gave an extract in former studies showing as high as 27.0 units of yellow and 0.9 units of red.

No opportunity was offered to investigate the yellow pigment which characterizes the tissue fat of the horse, but it may with safety be assumed to be carotin in view of the results of the studies made by the author with the cow and hen.

CONCLUSIONS.

A definite physiological relation exists in all species of animals between the pigmentation of tissue fat with carotinoids and the presence of these pigments in the blood serum. Species whose tissue fat is colored with the carotinoids, which include the cow, horse, and hen, carry the pigments in the blood serum. Species, the tissue fat of which is characterized by being colorless, including swine, sheep, and goats, carry only insignificant traces of the carotinoids in the blood serum even under the most favorable conditions.

An abundance of carotin in the blood serum and in the tissue fat is not a specific characteristic of the ruminants, as indicated by the absence of carotin from the blood serum and tissue fat of sheep and goats and its presence in the horse. The factors underlying the difference between these species in this respect offer an interesting field for study, as do those factors which cause the relatively exclusive resorption of carotin in the case of the cow and horse, and xanthophyll in the case of the hen.

THE RELATION OF THE UNIDENTIFIED DIETARY FACTORS, THE FAT-SOLUBLE *A*, AND WATER-SOLUBLE *B*, OF THE DIET TO THE GROWTH-PROMOTING PROPERTIES OF MILK.*

By E. V. McCOLLUM, N. SIMMONDS, and W. PITZ.

(*From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station, Madison.*)

(Received for publication, July 31, 1916.)

The results of nearly a thousand feeding experiments which we have conducted with our rat colony during the last few years all support the working hypothesis which we have explained and supported by experimental evidence in a number of previous papers.[1] This assumes the necessity of two dietary essentials as yet unidentified in the diet for growth or prolonged maintenance; *viz.*, the fat-soluble A of butter fat and certain other foodstuffs, and the water-soluble B which we have supplied by means of extracts prepared with water or alcohol from several foodstuffs.

These substances, or possibly groups of substances, which are indispensable from the diet during growth are of interest in relation to their possible formation within the maternal organism in connection with milk secretion. Experimental inquiry has not yet revealed the degree of dependence of animals upon a supply of these two dietary factors after growth has been completed. The evidence available would seem to point to the necessity of at least one of them, the water-soluble B, throughout life, for extracts which contain it induce relief of polyneuritis, and this may have its incidence at any time during life. The failure of rats to remain in a state of health on diets lacking the fat-soluble A we have pointed out in the papers cited.

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* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181, 231. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239.

The possibility still remains that the gonads, particularly in the female, or the mammary tissue, may be capable of producing one or both of these two dietary factors for the preservation of the young. The prevalence of infantile beri-beri among children whose mothers' diet is insufficient to prevent this disease may be raised against this assumption. The possibility still remains, however, that the diet of these people, which is restricted as to source and variety, and is of poor quality, may be inadequate with respect to the preventive substance itself and also as to its content of a possible precursor from which it might be formed through the peculiar property of a special organ in a manner analogous to the formation of the various hormones of internal origin.

In order to test this point it seemed desirable to observe the ability of normal female rats to suckle their young when confined to a diet which is known to be adequate for growth when satisfactory amounts of the unidentified A and B are added, and inadequate for growth when either of these factors is omitted. The paucity of such a diet in possible precursors is hardly to be expected and the results should enable us to decide whether these two essential substances pass into the milk only when they are furnished in the diet.

Method of Procedure.

Female rats were fed a ration of natural foodstuffs until they delivered their young. When the birth of young was observed the litter was in all cases reduced to four, in order that the nutritive undertaking for the mother in rearing them might not be excessive. Several series of experiments were conducted in which the basal ration consisted of purified casein, dextrin, inorganic salts, and agar-agar. It was adequate for growth except for the absence of the dietary factors A and B. In a second series the same ration plus butter fat (fat-soluble A) was employed. In a third series the water-soluble B only was added to the basal ration in the form of an alcoholic extract of wheat embryo, and in a fourth series both the fat-soluble A and the water-soluble

B were added. The results of feeding these rations to nursing mothers, as shown by their ability to induce growth in their young, are shown in Charts 3 to 6. For comparison we include also the records of rats fed a ration during the lactation which contained both the unidentified A and B but lacked protein.

DISCUSSION OF RESULTS.

The observation of Decaisne,[2] during the siege of Paris, that even during fasting or partial fasting vigorous young women were able to keep up a flow of milk sufficient to induce some growth in their infants, is in harmony with unpublished data obtained by Professor Hart of this laboratory. His data show that cows tend to keep up the flow of milk when the character of the diet is such as to induce a decided negative nitrogen balance, and that the product closely approximates the normal composition with respect to the constituents usually determined in analysis. This supports the view that the failure of the young to grow in certain of these experiments was not due to a depression of the milk supply, but to the production of milk which was not adequate for the promotion of growth on account of its deficiency in one or both of the unidentified factors we are considering.

It seems necessary to conclude that these two constituents of the diet pass into the milk only as they are present in the diet of the mother, and that milks may vary in their growth-promoting power when the diets of the lactating animals differ widely in their satisfactoriness for the growth of young.

It seems further justifiable to conclude that the chemical natures of the fat-soluble A and the water-soluble B, whether these represent in each case a single substance or a group of substances, are of such a character that they cannot be formed within the animal body from any of the cleavage products of proteins.

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[2] Decaisne, E., *Gaz. méd. Paris*, 1871, 317.

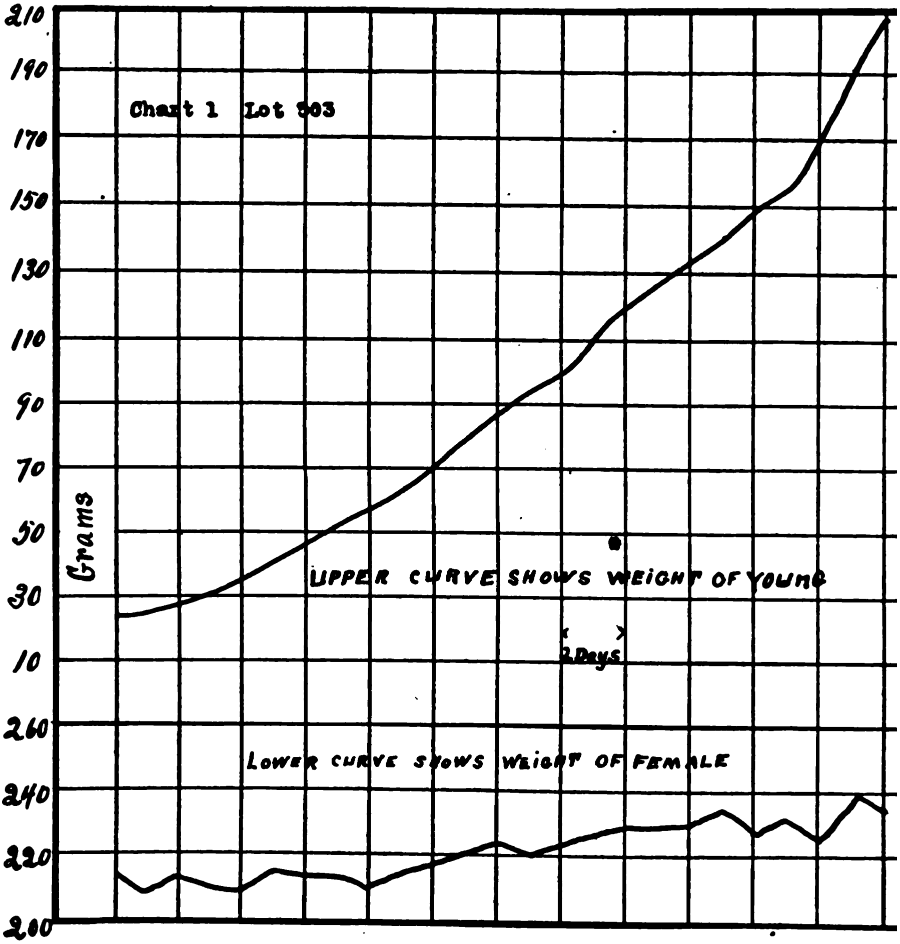

CHART 1.

Chart 1, Lot 503, illustrates the rate of gain of a litter of four young rats when the ration of the mother consisted of a highly satisfactory mixture of natural foodstuffs. The mother was able to increase her weight while the young were gaining at a remarkable rate.

We have described elsewhere the growth and reproduction records of rats confined to this diet.[3]

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[3] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 638.

The ration of this rat consisted of:

Casein	10.0
Milk powder	10.0
Wheat	64.0
Salt mixture (211)	3.6
Dextrin	7.4
Butter fat	5.0

Composition of Salt Mixture 211.	
NaCl	0.520
$CaH_4(PO_4)_2H_2O$	0.276
Ca lactate	1.971
K citrate	0.799
Fe acetate	0.100

Chart 2, Lot 640, Period 1, illustrates the extent to which the mother can sacrifice her own tissues for the preservation of the young when her diet contains all the essential factors except protein, and shows the rapid loss in weight of the mother and the slow growth of the young. Upon the addition of 18 per cent of casein in Period 2 after 5 days and 9 days respectively on the protein-free diet, they were able to increase perceptibly the rate of growth of the young and practically began to maintain their own body weights.

The ration in Period 1 (Lot 640) consisted of:

Dextrin	89.3
Salt mixture (185)	3.7
Butter fat	5.0
Agar-agar	2.0

Composition of Salt Mixture 185.	
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2H_2O$	0.540
Ca lactate	1.300

The dextrin carried the alcoholic extract of 10 gm. of wheat embryo. In Period 2, 18 per cent of casein replaced an equivalent amount of dextrin.

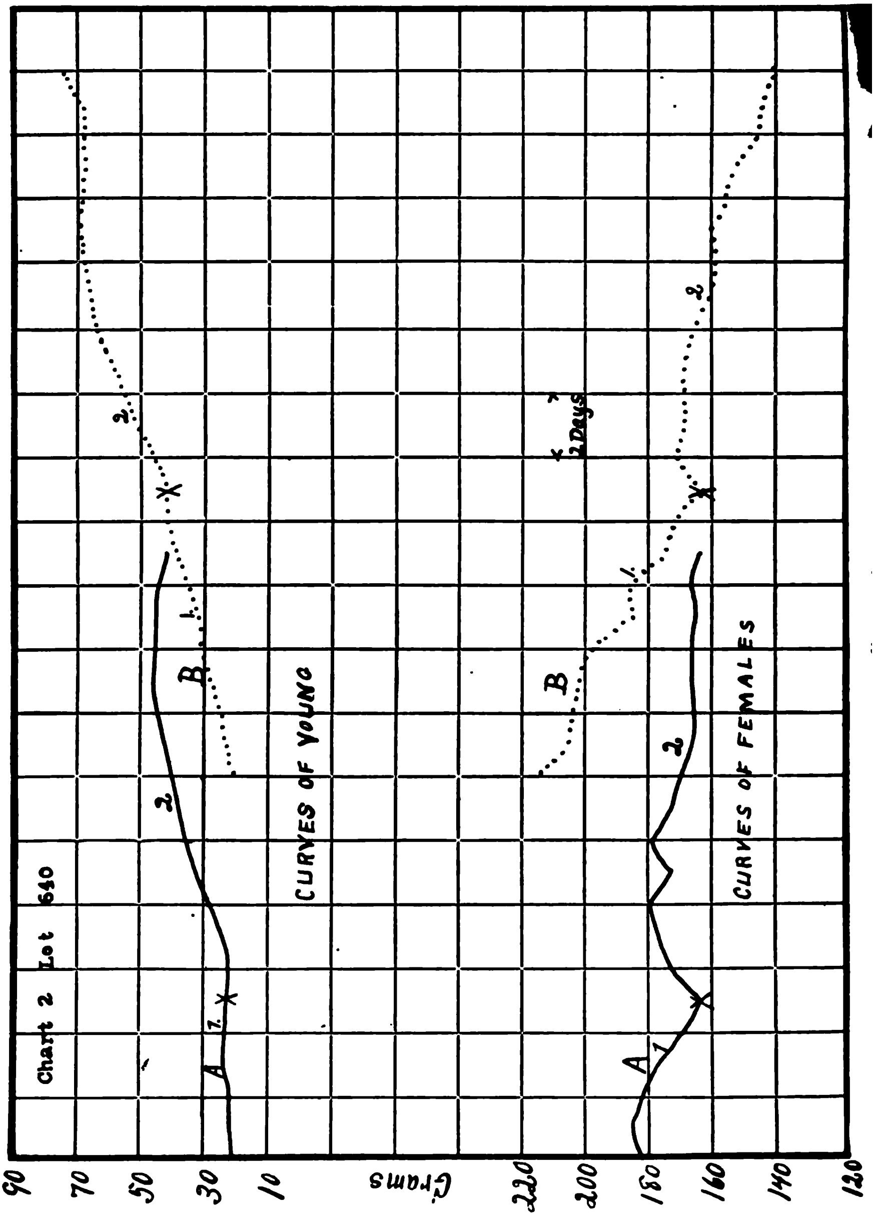


Chart 2 Lot 640
CURVES OF YOUNG
CURVES OF FEMALES
A
B
1
2
2 Days
Grams
90
70
50
30
10
220
200
180
160
140
120

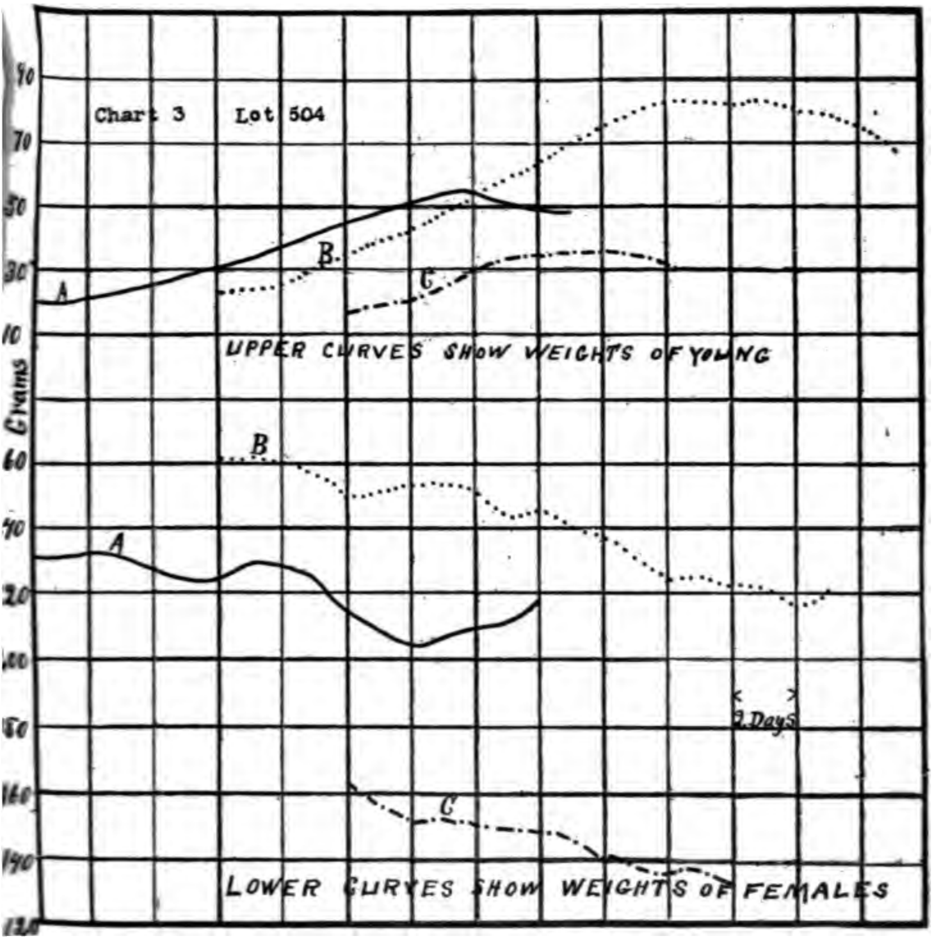


Chart 3 Lot 504
90
70
50
30
10
Grams
60
40
20
00
80
160
140
120
A
B
C
UPPER CURVES SHOW WEIGHTS OF YOUNG
B
A
9 Days
C
LOWER CURVES SHOW WEIGHTS OF FEMALES

CHART 3.

Chart 3, Lot 504, illustrates the degree to which the mothe can induce growth in the young when the diet contains an adequate amount of protein, carbohydrate, and inorganic salt Without the unidentified dietary factors, the fat-soluble A an water-soluble B in the food, the milk of the mother does no induce growth in the young except as she sacrifices her own tissu for their preservation. She is unable to do this beyond a limite extent. Composition of the ration, Lot 504:

Casein	18.0
Salt mixture (185)	3.7
Dextrin	76.3
Agar-agar	2.0

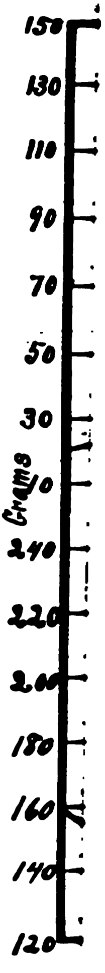
For the composition of Salt Mixture 185 see above.

Chart 4, Lot 506, shows Rats B and D which received a foo mixture which lacked only an alcoholic extract of a natural foo to supply the unknown dietary B. The increase in the weigh of the young was about the same as that seen when the mother diet lacked the protein factor. Rat A kept her young growing slowly during 16 days without the factor B in her diet, whe growth ceased. On changing her to Ration 503 (Chart 1) whic contained both milk powder and wheat, the young began t grow very rapidly.

Rat C was not so vigorous an animal and induced growth i her young only at the expense of decided loss of her own bod tissue. The addition of both the unidentified factors by th inclusion of butter fat and an alcoholic extract of wheat embry did not improve the quality of her milk to a very great degre but checked the rate of decline of the mother. When given Ratio 503 (Chart 1) containing both the A and B in liberal amount together with better proteins, the young began to grow on the 3r day after the change. Composition of the ration, Lot 506:

Casein	18.0
Dextrin	71.3
Salt mixture (185)	3.7
Butter fat	5.0
Agar-agar	2.0

For the composition of Salt Mixture 185, see above. Period 2, Rat was given Ration 503. See Chart 1. Rat C. The dextrin of her ratio carried the alcoholic extract of 10 gm. of wheat embryo. Period 3, Ra C was given Ration 503. See Chart 1.


Grams

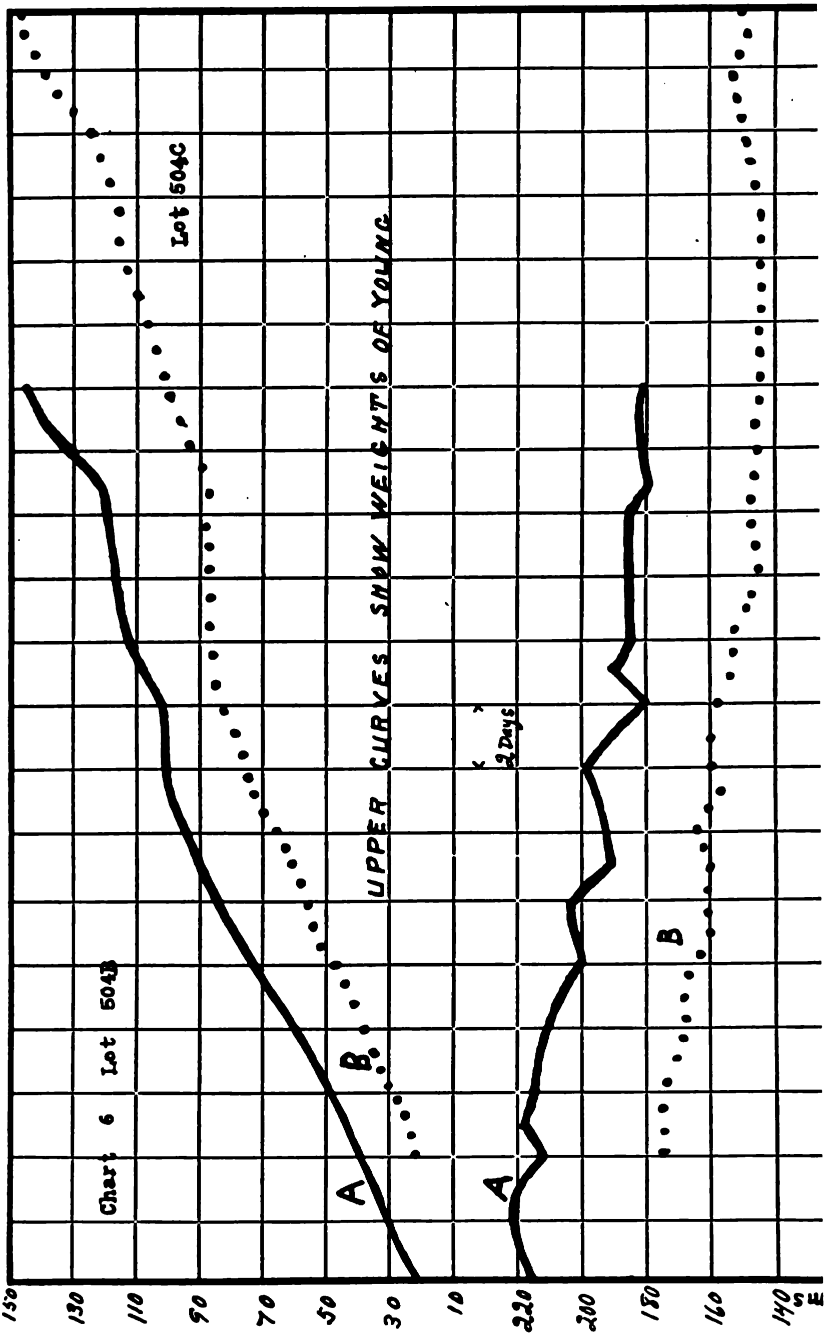


Chart 6 Lot 504B
Lot 504C
UPPER CURVES SHOW WEIGHTS OF YOUNG
A
B
A
B
2 Days
150
130
110
90
70
50
30
10
220
200
180
160
140
S
E

Chart 6, Lot 504 B, Rat A, shows fairly satisfactory growth in the young and practically maintenance of body weight of the mother on our basal ration to which both the fat-soluble A and water-soluble B were added at the beginning of the nursing period. The failure of the young in these two litters as well as those in Chart 5, Rat A, to grow at the maximum rate is probably to be attributed to the inadequacy of 18 per cent of casein as a source of protein for the lactating rat.

Rat B, Lot 504 C, whose ration was similar to that of Rat A except that double the amount of alcoholic extract of wheat embryo was contained in it, failed to promote the growth of her young any better because of the high content of the dietary factor B, indicating that the extract of 10 gm. of embryo which was contained in other rations of this series, supplied an adequate amount of this substance.

The ration (Lot 504 B) consisted of:

Casein	18.0
Dextrin	71.3
Salt mixture (185)	3.7
Butter fat	5.0
Agar-agar	2.0

The dextrin carried the alcoholic extract of 10 gm. of wheat embryo.

For the composition of Salt Mixture 185, see above.

For Lot 504 C the ration was the same as that of 504 B except that the dextrin carried the alcoholic extract of 20 gm. of wheat embryo.

THE INFLUENCE OF ETHER ANESTHESIA ON AMINO-ACIDS OF BLOOD SERUM.

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(Received for publication, August 7, 1916.)

The pharmacology of ether used as an anesthetic is the subject of many conflicting theories and statements. The numerous phases of the subject still need minute and extensive investigation before any clear conception of the action of this much used compound can be secured. We have already made studies of the dextrose and diastase content of the blood as affected by ether anesthesia.[1] In this case the amino-acid content of the blood serum has been the subject of investigation.

EXPERIMENTAL WORK.

Five groups of dogs were used in this work. Each group was treated differently. Group I was fed ground lean beef daily for 1 week. Each dog received 250 gm. about noon each day. The operative work was done on the dogs the day after the last feeding. A sample of blood was taken from the jugular vein just before anesthesia. The ether was given by forcing air saturated with ether into a nose mask for the dog. After surgical anesthesia had continued for 15 minutes another sample of blood was taken. The amino-acid content of the blood was determined by the method of Van Slyke[2] with the modification that the sample and reagents were increased five times and the determinations made on 2 cc. volume of the total filtrate which had been made up to 10 cc. volume. Several determinations were made on each sample and the average was taken as the correct result. The results of the work on Group I are given in Table I.

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[1] Ross, E. L., and McGuigan, H., *J. Biol. Chem.*, 1915, xxii, 407.

[2] Van Slyke, D. D., Vinograd-Villchur, M., and Losee, J. R., *J. Biol. Chem.*, 1915, xxiii, 385.

TABLE I.

Influence of Ether after a Week of Meat Feeding.

Dog.	Mg. of amino N per cc. of serum.		
	Before ether.	After 15 min. of anesthesia.	Change.
1	0.066	0.064	−0.002
2	0.046	0.049	+0.003
3	0.069	0.063	−0.006
4	0.111	0.110	−0.001
5	0.111	0.101	−0.010
Average.......	0.0806	0.0774	−0.0032 −4.0 per cent

Group II was made up of five dogs which were fed on a low protein diet for 1 week previous to the test. They were fed 200 gm. of a mixture of crackers 2 parts and lard 1 part. The animals otherwise were treated in the same way as those of Group I. The results are given in Table II.

TABLE II.

Influence of Ether after a Week of Low Protein Diet.

Dog.	Mg. of amino N per cc. of serum.		
	Before ether.	After 15 min. of anesthesia.	Change.
1	0.070	0.074	+0.004
2	0.022	0.024	+0.002
3	0.037	0.034	−0.003
4	0.063	0.063	−0.000
5	0.049	0.046	−0.003
Average.......	0.0482	0.0482	−0.000 0.0 per cent

Group III consisted of five dogs which had not been receiving a special diet before the tests. Half an hour before bleeding the first time each dog was given a mixture containing 100 gm. of sugar, 16 gm. of starch, and water. After bleeding they were anesthetized and treated as were the dogs of the preceding groups. The results are given in Table III.

TABLE III.

Influence of Ether ½ Hour after Carbohydrate Feeding.

Dog.	Mg. of animo N per cc. of serum.		
	Before anesthesia.	After 15 min. of anesthesia.	Due to ether.
1	0.072	0.069	−0.003
2	0.054	0.055	+0.001
3	0.069	0 061	−0.008
4	0.062	0.067	+0.005
5	0.068	0.070	+0.002
Average.......	0.065	0.0644	−0.0006 −0.9 per cent

Group IV consisted of ten dogs which had not been previously dieted. They were bled, fed 200 gm. of ground lean beef each, bled at the end of half an hour after feeding, anesthetized, and after 15 minutes of surgical anesthesia, bled again. Amino-acid determinations were made on the samples. The results are given in Table IV.

TABLE IV.

Influence of Ether ½ Hour after Meat Feeding.

Dog.	Mg. of amino N per cc. of serum.			Changes.	
	Before feeding.	½ hr. after feeding.	After anesthesia.	Due to feeding.	Due to ether.
1	0.061	0.061	0.051	0.000	−0.010
2	0.123	0.112	0.116	−0.011	+0.004
3	0.071	0.072	0.057	+0.001	−0.015
4	0.118	0.130	0.124	+0.012	−0.006
5	0.046	0.051	0.041	+0.005	−0.010
6	0.149	0.127	0.138	−0.022	+0.011
7	0.049	0.046	0.038	−0.003	−0.008
8	0.121	0.118	0.130	−0.003	+0.012
9	0.071	0.076	0.062	+0.005	−0.014
10	0.144	0.140	0.140	−0.004	0.000
Average..	0.0953	0.0933	0.0897	−0.0020 2.1 per cent	−0.00 6 3.9 per cent

Group V was made up of undieted dogs. They were bled, fed a pound of ground lean beef each, bled after 4 hours, anesthetized, and bled again after 15 minutes of anesthesia. The results are given in Table V.

TABLE V.

Influence of Food and Ether.

Dog.	Mg. of amino N per cc. of serum.			Changes.	
	Before feeding.	4 hrs. after feeding.	After 15 min. anesthesia.	Due to feeding.	Due to ether.
1	0.116	0.168	0.160	+0.052	−0.008
2	0.105	0.159	0.148	+0.054	−0.011
3	0.091	0.136	0.144	+0.045	+0.008
4	0.081	0.179	0.135	+0.098	−0.044
5	0.097	0.141	0.124	+0.044	−0.017
Average..	0.098	0.1566	0.1422	+0.0586 +59.8 per cent +47.8 per cent*	−0.0144 −9.2 per cent −4.6 per cent*

*Averages with the omission of Dog 4, in which there was an exceptionally large increase due to feeding, and a correspondingly large decrease due to ether.

DISCUSSION.

The results given in Table I show the influence of ether anesthesia on the amino-acid content of the blood serum of dogs which had been dieted for 1 week on lean beef. There was a small drop in four out of five cases. The average change in the group was a drop of 4.0 per cent of the original content of the serum.

The change in the amino-acid content of the blood serum due to anesthesia of dogs fed on practically no protein for 1 week is given in Table II. In one case there was no change, in two there was an increase, and in two a decrease. The average of all five changes was zero.

The figures given in Table III show the change, due to ether, in the amino-acid content of blood serum of dogs which half an hour previous to anesthesia received considerable amounts of carbohydrates. The results are variable. Three out of five show an increase due to ether but the average of all shows a decrease of 0.9 per cent. Compared with the wide individual variations, the average change is negligible.

Table IV gives the results of feeding meat half an hour before anesthesia. The change in the amino-acid content of the blood serum due to the taking of food and to the administration of ether is determined. The individual changes due to both food and ether are variable; some are positive and some are negative. The average change due to meat feeding was a fall of 2 per cent and that due to ether anesthesia following a fall of 3.9 per cent.

Table V contains data on the amino-acid change of the blood serum due first to 4 hours of meat digestion and second to ether anesthesia following. The average change for all five dogs due to meat digestion was 59.8 per cent increase and that due to ether anesthesia was a fall of 9.2 per cent. Dog 4 made an enormous increase due to feeding and a very exceptionally large decrease due to the ether. Averaging the results of the other four dogs, there was an increase due to meat digestion of 47.8 per cent and a decrease due to anesthesia of 4.6 per cent. This probably is more near the changes that would be found in the average normal dog. Van Slyke and Meyer[3] obtained similar results.

It is interesting to note that very roughly the amount of decrease of amino-acids due to ether anesthesia follows the initial amino-acid content of the blood. This is shown in the following table compiled from the preceding tables:

Table.	Amino N before ether.	Fall.
		per cent
II	0.0482	0.0
III	0.0650	0.9
IV	0.0953	3.9
I	0.0806	4.0
V	0.0980	4.6–9.2

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[3] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 409.

CONCLUSIONS.

The amino-acid content of the blood serum of dogs is affec
as follows. 1. Not materially changed in half an hour by fe
ing either carbohydrates or meat. 2. Increased considera
in 4 hours after feeding a pound of meat to each dog. 3.
materially decreased by 15 minutes of ether anesthesia, fi
after a week of meat diet, second, after a week of low pro
diet, third, half an hour after feeding meat, or fourth, half
hour after feeding carbohydrates and fat. The decrease at
is not above 4.0 per cent. 4. Decreased 4.6 to 9.2 per cent a
an amino-acid rise due to 4 hours of meat digestion.

STUDIES ON GROWTH.

III. THE COMPARATIVE VALUE OF LARD AND BUTTER FAT IN GROWTH.

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(Received for publication, August 3, 1916.)

In our first communication[1] of this series, we advanced the opinion that we were dealing with a problem very similar to, if not identical with, beri-beri. Our main objective was to ascertain the simplest dietary conditions necessary to enable a young rat to reach maturity. Subsequently we found[2] that artificial diets containing butter, without yeast or similar vitamine-containing substances, are insufficient to promote growth in young rats; and at that time the question whether butter could be replaced by lard with the same ultimate success was left open. We have carried out experiments of longer duration, employing both lard and butter as the fat fraction of these diets, and submit results which enable us to form a more definite opinion as to the relative value of these two fats.

Our experience demonstrates that there are wide variations depending on the constitution of the individual rats. Every rat taken indiscriminately is not suitable for this class of work. As a matter of fact in experiments carried out in Toronto 80 per cent of the rats purchased from dealers were rejected on account of physical defects not apparent before the initiation of the experiment. A second complication is a diminished resistance to infection, which follows the use of all artificial diets. The meager knowledge we possess of the pathological conditions in rats may lead to a condemnation of the diet; whereas actually the condition could be remedied without change of diet, if we were able to recognize its nature. As an example of this, rats on artificial diets frequently contract an eye infection which can be treated with a certain degree of success by an application of a few drops of zinc sulfate solution. If untreated this condition is accompanied by loss in weight, becomes acute, and terminates fatally.

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[1] Funk, C., and Macallum, A. B., Jr., *Z. physiol. Chem.*, 1914, xcii, 13.

[2] Funk and Macallum, *J. Biol. Chem.*, 1915, xxiii, 413.

The first series of experiments were carried out on diets containing lard as the fat component, and dried powdered yeast. Rats on this diet grew normally for 60 to 90 days, but eventually displayed symptoms (bleeding from the eyes, nose, and ears, petechiæ and hemorrhages under the skin of the tail) which might be regarded as scorbutic. This terminated fatally if no change of diet was effected. When moist yeast was substituted for the dried preparation the rats could be kept for 150 days and attained approximately adult size. Autolyzed yeast was equally efficient in this respect. Similar results were obtained on addition of orange juice to the drinking water, although orange juice itself has neither growth-promoting nor maintaining properties, unless supplemented by yeast.

Diets in which butter partially or wholly replaces lard have a slight superiority over those containing lard, which is more than can be explained by the antiscorbutic properties of the butter. Rats on yeast and butter diets often show the eye affection regarded by most of the investigators as characteristic of dietary deficiencies, and we are convinced that none of the artificial diets so far investigated can be compared with a normal dietary in its efficiency for growth. This deficiency introduces an additional complication and must be taken into account in subsequent investigations.

EXPERIMENTAL.

The methods of preparing the diets were very much the same as those described in our earlier publications. The experiments varied slightly as to their conditions in New York and Toronto but the ultimate results were identical. The charts and tables are representative of the different groups of experiments.

Experiment I.

Rats 49, 50, 51, and 52 (Fig. 1) were kept on diets containing dried yeast and lard for about 68 days. At that time a deficiency was noticed which, in previous experiments, led to the death of all the rats and could not be corrected by a larger supply of dried yeast. Then fresh moist pressed yeast was substituted, the deficiency disappeared, and the rats attained approximately adult weight.

A second series, Rats 61 and 62 (Fig. 2), were placed on diets containing lard for 44 days, being changed to a diet of butter and dried yeast after this period. No increment in growth was noticed as the result of this change.

In the butter experiments Rats 53 and 54 (Fig. 1) have also shown a marked improvement on changing the yeast from the dried to the moist form, more especially as regards their external appearance. Rats 47 and 48 (Fig. 2) were kept on diets with butter and dried yeast and these have also developed symptoms which persisted when the diets were substituted by lard and autolyzed yeast. Rats 59 and 60 (Fig. 2), exceptionally healthy specimens, were kept on butter-containing diet for 44 days and then changed[3] to diet with lard and autolyzed yeast, for a longer period than was indicated in the chart, without the rate of growth being modified. On several occasions rats showing deficiency on a lard-containing diet were placed on a butter diet with the hope of relieving the symptoms. The improvement which resulted from this change was only temporary and several rats died after being kept 30 days on butter.

In all the experiments a marked improvement resulted when a diet of a different composition was given or even from a fresh preparation of the same diet. This might indicate that the diets lose part of their nutritive value when stored for lengthy periods.

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[3] Encircled numbers on Fig. 2 indicate the point at which that diet was begun.

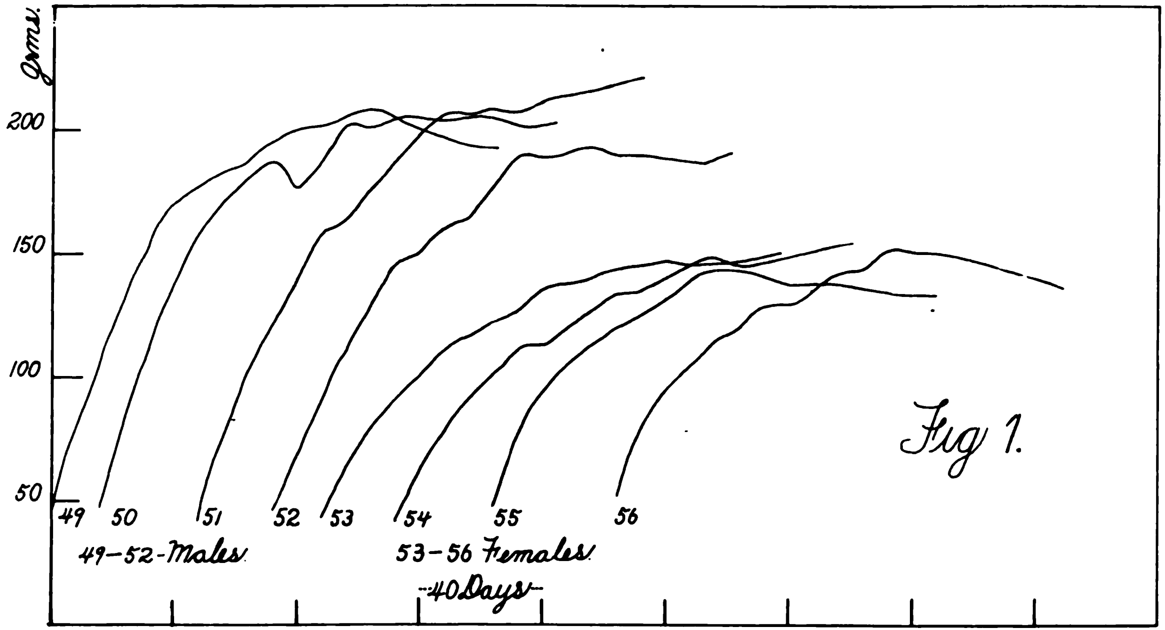

FIG. 1. Rats 49, 50, 51, and 52 were kept on diets containing lard and dried yeast. The animals recovered when moist yeast was substituted for dried yeast. Rats 53 and 54 were kept on butter and dried yeast. Here also a marked recovery was noticed on changing to the wet form of yeast. Rats 55 and 56 were kept on a diet containing casein purified according to the method of McCollum; no advantage of the use of this method is noticeable.

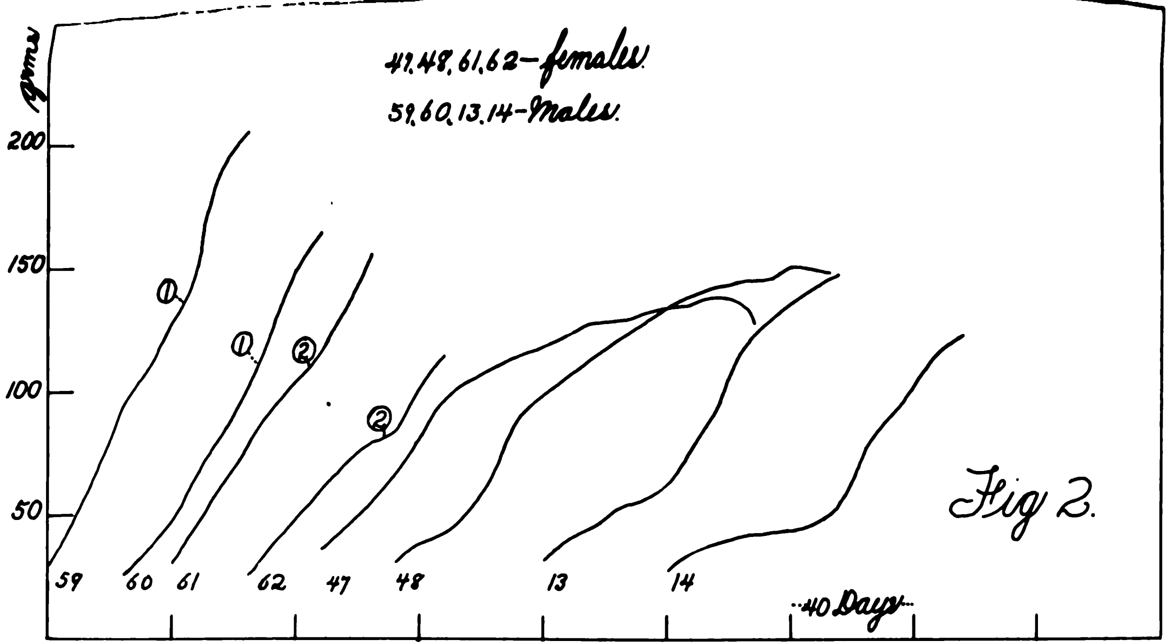

Fig. 2. Rats 59 and 60 have been kept on a diet of butter and dried yeast, which diet was then changed at the point indicated in the curve to lard and autolyzed yeast without any effect on the rate of growth. Rats 61 and 62 were kept on lard and then changed to butter without any effect on growth. Rats 47 and 48 were kept on butter and dried yeast and developed signs of food deficiency which persisted when the diet was changed to lard and autolyzed yeast. Rats 13 and 14 were kept on a diet containing casein which had been autoclaved. The rats failed to grow on this diet but recovered when 1 cc. of orange juice was added, which indicates that this deficiency was not due to chemical changes in the casein.

Diets (Gm.).

	1.	2.	3.	4.	5.
Casein	22	22	22	22	22
Sugar	10	10	10	10	10
Starch	30	27	27	29	27
Lard	30	30	30	30	30
Salt	3	3	6	6	6
Agar	2	2	2	2	2
Yeast (dry)	3	6	3		
Yeast (moist) equal to				1 of dry yeast.	3 of dry yeast.

Rats 49 and 50. Males.
0– 68 days Diet 2
69– 98 " " 4
99–150 " " 5

Rats 51 and 52. Males.
0– 52 days Diet 1
53– 68 " " 3
69– 98 " " 4
99–150 " " 5

Days.	Weight.		Average food intake per day.	Weight.		Average food intake per day.
	49.	50.		51.	52.	
	gm.	*gm.*	*calories*	*gm.*	*gm.*	*calories*
0	44	46		41	46	
4	55	58	77.4	62	55	78.2
8	81	85	92.4	72	65	81.3
12	96	100	103.2	91	82	67.2
16	104	110	105.7	102	91	97.1
20	119	124	110.3	110	100	102.2
24	132	137	110.5	119	110	106.8
28	146	150	110.0	132	120	100.3
32	150	158	111.0	138	128	100.8
36	162	163	109.3	148	138	112.2
40	170	174	114.6	159	147	116.2
44	173	176	114.3	160	150	115.2
48	177	180	111.1	163	150	117.5
52	178	184	95.4	170	158	108.6
56	182	188	110.8	176	161	120.3
60	185	191	103.8	178	163	106.6
64	186	176	78.7	175	164	96.9
68	184	182	90.0	192	167	120.7
76	199	200	115.9			116.3
84	201	201	98.5	206	190	108.5
100	207	205	85.0	209	192	91.0
120	200	205	105.7	210	190	103.7
140	193	201	76.3	216	186	85.4
150	192	203	75.8	221	190	67.4

Diets (Gm.).

	1.	2.	3.	4.
in................	22	22	22	22
r..................	10	10	10	10
:h................	30	27	29	27
er fat............	18	18	18	18
....	12	12	12	12
....	3	3	6	6
....	2	2	2	2
t (dry)...........	3	6		
:t (moist) equal to			1 of dry yeast.	3 of dry yeast.

Rats 53 and 54. Females.
0– 52 days Diet 1
53– 68 " " 2
69–100 " " 3
101–150 " " 4

Days.	Weight.		Average daily food intake.
	53.	54.	
	gm.	*gm.*	*calories*
0	43	41	
4	54	55	74.3
8	64	63	66.2
12	78	75	72.0
16	80	80	77.0
20	85	87	73.5
24	90	92	76.8
28	96	95	81.1
32	100	101	79.2
36	106	106	88.9
40	112	112	87.3
48	112	112	87.2
56	119	116	83.7
64	123	126	95.2
84	138	135	79.8
100	144	146	76.5
120	149	147	79.2
150	147	153	92.8

Diets (Gm.)

	1.	2.	3.
Casein	22	22	22
Sugar	10	10	10
Starch	28	30	23
Butter	30	30	
Lard			30
Agar	2	2	2
Salts	2	2	2
NaHCO$_3$	1	1	1
Yeast (dry)		3	
Yeast (autolyzed) equal to	1.5 of dry yeast.		3 of dry yeast.

Rats 47 and 48. Females.
0– 16 days Diet 1.
16–123 " " 2.
123–140 " " 3.

Days.	Weight.		Average daily food intake.
	47.	48.	
	gm.	gm.	gm.
0	36.5	31	
4	41	34	6.2
8	45.5	39	9.5
12	51.5	43	9.1
16	54.5	42.5	9.1
20	58	45	10.0
24	67.5	50.5	11.2
28	76	56.5	12.1
32	85	66	14.1
36	91	78	13.6
40	99	91	14.2
44	102	93	12.5
48	105	98	13.5
52	108	100.5	14.5
56	111	107	14.3
60	114.5	113.5	15.0
64	115.5	115	16.9
68	119	122	15.2
72	119	120	14.0
76	121.5	125.5	15.3
80	124.5	129.5	15.3
84	127	134.5	17.1
88	128	134.5	14.6
92	126.5	135	14.1
96	128.5	139	16.1
100	130.5	141	14.7
108	134	144.5	16.6
116	133	145	16.9
124	135	146.5	16.6
132	139.5	154	14.6
140	128	149.5	14.0

Diets (Gm.).

	1.	2.
Casein	22	22
Starch	23	30
Sugar	10	10
Butter		30
Lard	30	
Agar	2	2
Salts	2	2
$NaHCO_3$	1	1
Yeast (dry)		3
Yeast (autolyzed) equal to	3 of dry yeast.	

Rats 59 and 60. Males.	Rats 61 and 62. Females.
0–44 days Diet 2.	0–44 days Diet 1.
45–64 " " 1.	45–64 " " 2.

Days.	Weight.		Average daily food intake.	Days.	Weight.		Average daily food intake.
	59.	60.			61.	62.	
	gm.	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*
0	30.5	25	6.7	0	30.5	25.5	6.6
4	40	31	8.2	4	38	32	9.3
8	47.5	35.5	9.7	8	46.5	38	9.3
12	59	42.5	11.2	12	55.5	43	10.7
16	70	48	13.8	16	62.5	48	12.5
20	81.5	58.5	14.8	20	71.5	55	12.6
24	94	68	16.2	24	80.5	61.5	14.6
28	106	75.5	17.2	28	87.5	67	14.2
32	114	82.5	20.1	32	93.5	71	16.1
36	127.5	90	20.8	36	99.5	76	14.9
40	139.5	100.5	23.8	40	105	80.5	14.2
44	155	113	24.9	44	111.5	84.5	14.2
48	167	124	23.7	48	115	83	16.6
52	180	138	21.8	52	123	96	17.6
56	192	150	22.0	56	132	100	15.2
60	199	157	21.1	60	142	104.5	17.1
64	205	165	21.1	64	156.5	115	

Experiment VI.

The casein preparation used in this series was purified by washing, following the method of McCollum and Davis.[4] In this paper the authors claim that purification of casein by bo ing with alcohol destroys some of the amino-acids and results loss of its nutritive properties. The results in this case (Ra 55 and 56, Fig. 1) were identical with those which were obtain with casein purified by extraction with hot alcohol. This latt method was used in purifying the casein in the first experime

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[4] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231.

Diets (Gm.).

	1.	2.	3.
Casein (McCollum)	22	22	22
Sugar	10	10	10
Starch	27	29	27
Lard	30	30	30
Salt	3	6	6
Agar	2	2	2
Yeast (dry)	6		
Yeast (moist) equal to		1 of dry yeast.	3 of dry yeas

Rats 55 and 56. Females.
0- 59 days Diet 1.
60- 87 " " 2.
88-140 " " 3.

Days.	Weight.		Average daily food intake.
	55.	56.	
	gm.	*gm.*	*calories*
0	48	52	
4	67	71	75.1
8	80	82	92.2
12	89	91	88.4
16	96	96	77.2
20	104	102	86.0
24	107	104	74.9
28	109	109	94.3
32	115	116	79.8
40	120	120	88.4
60	140	133	86.3
80	144	143	78.4
100	136	150	79.9
140	133	136	61.9

nother series, of which Rats 13 and 14 (Fig. 2) are representa-
s, received casein which had been autoclaved for 1 hour at 15
nds' pressure, according to McCollum and Davis. On this diet
rats failed to grow, but after 28 days 1 cc. of fresh orange
e was added, and normal growth was resumed. It seems
able that the impaired value of heated casein is not due so
h to the destruction of amino-acids as to the loss of its anti-
butic properties.

Diets (Gm.)

	1.	2.
in	22	22
ar	10	10
ch	30	29
d	30	30
s	3	3
r	2	2
st (dry	3	4

Rats 13 and 14. Males.
0– 14 days Diet 1.
15– 96 " " 2.
28–100 " 1 cc. orange juice.

Days.	Weight.		Average daily food intake.
	13.	14.	
	gm.	gm.	gm.
0	33.3	27.2	
4	40.1	33.7	6.2
8	41.4	37.1	6.0
12	44.6	39.8	7.1
16	46.4	39.4	7.9
20	50.6	41.4	7.2
24	54.3	42.0	8.1
28	54.6	41.5	6.5
32	55.1	42.0	4.7
36	63.1	47.0	8.2
40	62.1	44.0	6.8
44	70.8	46.2	8.4
48	77.1	46.2	10.6
52	87.1	51.8	12.2

Rats 13 and 14. Males—*Continued.*

Days.	Weight.		Average daily food intake.
	13.	14.	
	gm.	*gm.*	*gm.*
56	93.0	54.6	12.4
60	102.0	64.2	12.9
64	119.3	78.6	15.6
68	126.0	89.3	15.1
72	130.0	91.6	15.4
76	130.9	98.6	14.9
80	137.4	103.8	15.1
84	140.0	112.4	14.8
88	144.7	117.0	11.2
92	145.0	119.8	14.2
96	147.0	123.0	11.4

SUMMARY.

The failure of rats to grow on a lard and yeast diet is partially due to the development of scorbutic symptoms. These can be relieved to a marked degree by using moist instead of dried yeast and still more so by using moist yeast and butter. Even in the latter case the existing deficiencies are not entirely corrected, since many rats decline on this diet. Rats which fail on lard do not always recover on a diet containing butter. It seems also possible that yeast on account of its high content in purines, and perhaps other constituents, is not an ideal addition in experiments of long duration, even in spite of its marked growth-promoting power. The impaired nutritive value of heated casein does not seem to be due to destruction of amino-acids but to destruction of vitamines.

STUDIES ON GROWTH.

IV. THE ACTION OF YEAST FRACTIONS ON THE GROWTH OF RATS.

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The close relationship existing between the beri-beri and owth problems suggests the possibility of a fractionation of e active substance along lines already used in the investigation beri-beri. Accordingly phosphotungstic acid was selected r the first attempt to separate out a physiologically active action which would stimulate the growth of young rats. The perimental difficulties which have repeatedly been emphased in the investigation of the beri-beri vitamine, due to inability of this substance, were also encountered in the study growth. The physiological activity of the different fractions minishes with each manipulation and both the problems of ri-beri and of growth will not be solved until more suitable ethods for the isolation of vitamine are available.

The results obtained so far clearly indicate that the growth-omoting substance is analogous to and possibly identical with e beri-beri vitamine and can be almost entirely precipitated ith phosphotungstic acid. Subsequent fractionation of the resue obtained from the decomposed precipitate with silver nitrate d also with silver nitrate and baryta has shown that the recipitate containing purine bases and the filtrate from the lver nitrate and baryta precipitation are entirely negative as their growth-promoting action; whereas the substances precipitated with silver nitrate and baryta possess traces of the activity of the initial phosphotungstic acid precipitate. The experimental evidence indicates that considerably larger quantities of vitamines are necessary for stimulating growth than for curing beri-beri, and the losses occurring during fractionation are more apparent in the former than in the latter case. However, it must be admitted that while it is uncertain whether these two substances are chemically different, the results obtained do not exclude such a possibility. Lloyd's reagent, as recommended by Seidell,[1] has also been used as a precipitant without much success, as the rats on the filtrate have also shown increments in growth.

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In the first instance autolyzed yeast was slightly acidified with hydrochloric acid and completely precipitated with phosphotungstic acid, carefully avoiding an excess of this reagent. After allowing the mixture to stand for 24 hours, the precipitate was collected on a Buchner funnel and repeatedly washed with a cold solution of phosphotungstic acid containing hydrochloric acid. The precipitate was then decomposed by the method described by Van Slyke,[2] with a mixture of amyl alcohol and ether and hydrochloric acid, only a small quantity of the precipitate remaining unchanged. After filtration of this small fraction the aqueous extract was evaporated *in vacuo* and the residue made up to a known volume and mixed in the diet in quantities calculated from the amount of autolyzed yeast necessary to promote growth. However, the quantity of this fraction had to be doubled and even tripled in order to obtain satisfactory results. The phosphotungstic acid filtrate was worked out in a similar way. This process offers the advantage that the yeast fraction is completely freed from substances soluble in lipoid solvents. The purine fraction was obtained from the phosphotungstic acid precipitate fraction by precipitation with silver nitrate and subsequent decomposition with sulfuretted hydrogen. The filtrate from the purine bases was precipitated with baryta in the usual way and the precipitate decomposed, freed from traces of baryta, evaporated, and the residue mixed with the diet. The filtrate from the fraction containing vitamines was reprecipitated with phosphotungstic acid and the precipitate obtained after thorough washing, decomposed with amyl alcohol and ether. The results with the purine fraction and also with the silver nitrate-baryta filtrate are not included in this paper as they were entirely negative. The effect of the silver nitrate-baryta fraction was not sufficiently marked to encourage further investigation. The diet contained lard as the fat constituent, and 1 per cent sodium bicarbonate was added to neutralize the hydrochloric acid present in this fraction. Orange juice to the extent of 1 cc. a day was added to the drinking water to prevent the onset of scorbutic symptoms.

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[1] Seidell, A., *U. S. Public Health Report*, No. 325, 1916.

[2] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxii, 281.

A large number of rats were kept on the above diets, especially on the phosphotungstic precipitate and filtrate and the records of only a few were selected for publication, in order to save space. Rats 9 and 10 were kept on phosphotungstic acid filtrate throughout the experiment. Rats 11 and 12 were changed after 34 days to the diet containing the phosphotungstic precipitate fraction which was followed by an improvement warranting the view that the growth-promoting substance is contained in this precipitate (Fig. 2 b).

Diets (Gm.).

	1.	2.
Casein	22	22
Sugar	10	10
Starch	23	23
Lard	30	30
Agar	2	2
Salts	2	2
$NaHCO_3$	1	1
Phosphotungstate precipitate		10
" filtrate	10	

Rat 9 (male) and Rat 10 (female).
0–96 days Diet 1.
28–100 " 1 cc. orange juice.

Rats 11 and 12. Males.
0–34 days Diet 1.
35–100 " " 2.
76–100 triple vitamine addition.

Days.	Weight.		Average daily food intake.	Days.	Weight.		Average daily food intake.
	9.	10.			11.	12.	
	gm.	gm.	gm.		gm.	gm.	gm.
0	35.2	41.8		0	37.9	34.2	
4	42.8	47.3	9.5	4	46.2	41.8	7.6
8	43.0	47.3	7.8	8	45.8	44.2	5.5
12	44.6	49.3	7.0	12	42.8	45.3	7.6
16	46.8	55.4	8.7	16	45.3	45.8	7.8
20	46.2	56.6	7.5	20	46.9	45.7	7.7
24	46.6	54.7	7.8	24	46.2	46.5	6.6
28	46.5	52.8	5.5	28	44.3	45.0	5.4
32	42.9	49.9	7.4	32	40.4	39.4	4.7
36	37.8	41.6	7.8	36	39.8	37.2	5.6
40	36.8	41.6	7.7	40	50.5	48.2	9.1
44		41.8	6.6	44	52.2	50.0	8.9
48		43.9	5.3	48	54.8	57.0	8.0
52		44.0	5.3	52	58.0	60.7	8.7
56		43.8	3.8	56	63.5	67.3	11.0
60		41.6	3.5	60	66.3	71.0	12.4
64		44.6	3.5	64	66.1	70.3	8.8
68		41.0	3.2	68	70.6	75.7	11.1
72		43.5	3.5	72	75.0	82.6	9.9
76		42.9	3.4	76	76.0	84.8	11.9
80		45.0	3.0	80	77.6	91.1	11.1
84		42.2	3.1	84	75.0	85.2	9.6
88		44.1	2.7	88	79.4	90.0	8.8
92		43.0	3.2	92	78.5	90.9	4.4
96		41.0	2.9	96	74.7	91.7	6.4
100				100	70.0	76.3	6.4

A second series of experiments was carried out on pressed yeast which had been heated with 10 per cent sulfuric acid at 90–95° for 6 hours. The hydrolysate was filtered, diluted with an equal volume of water, and precipitated with phosphotungstic acid. After standing for 24 hours the precipitate was filtered at the pump and well washed with 5 per cent sulfuric acid.

The precipitate was decomposed in the ordinary way with baryta. The final solution, slightly acid, was neutralized with sodium carbonate, carefully avoiding an excess, distilled *in vacuo*, standardized, and definite quantities were added to the diet. The filtrate of the phosphotungstic acid precipitation was treated in a similar way.

The diet containing the substances precipitated by phosphotungstic acid was fed to four rats (Rats 80 to 83, Fig. 1) and enabled them to double their original weight after 32 to 36 days. This is about double the time required when yeast is the source of vitamines and the depreciation is due to the fractionation with the precipitating reagent.

Two rats (84 and 85, Fig. 2 a) were fed the diet containing the residue from the phosphotungstic filtrate. After 28 days they had added only a third to their original weight and had all the external symptoms of an acute deficiency. Then the diet with the precipitate was substituted and in 11 days they rapidly doubled their original weight and presented a normal appearance.

Diets (Gm.).

	1.	2.
Casein	22	22
Sugar	10	10
Starch	24	24
Fat (lard)	30	30
Salt	6	6
Agar	2	2
Residue phosphotungstic acid precipitate equal to	6	6 Dried yeast.
Residue phosphotungstic acid filtrate equal to		

Rats 80, 81, and 82. Males.
Rat 83. Female.
0–36 days Diet 1.

Days.	Weight.		Average daily food intake.	Weight.		Average daily food intake.
	80.	81.		82.	83.	
	gm.	*gm.*	*calories*	*gm.*	*gm.*	*calories*
0	24	25		20	30	
4	31	33	29.8	23.5	37	27.8
8	36	40.5	33.5	28	43	32.8
12	36	43	35.7	29	45	33.3
16	38	44.5	40.5	29	48	40.5
20	40	48	28.6	31	51	38.3
24	42	49	30.1	34	54	30.2
28	44.5	52	27.8	38	57	35.0
32	46	54	28.7	40	59	27.4
36	49	56	25.0	42.5	61	29.8

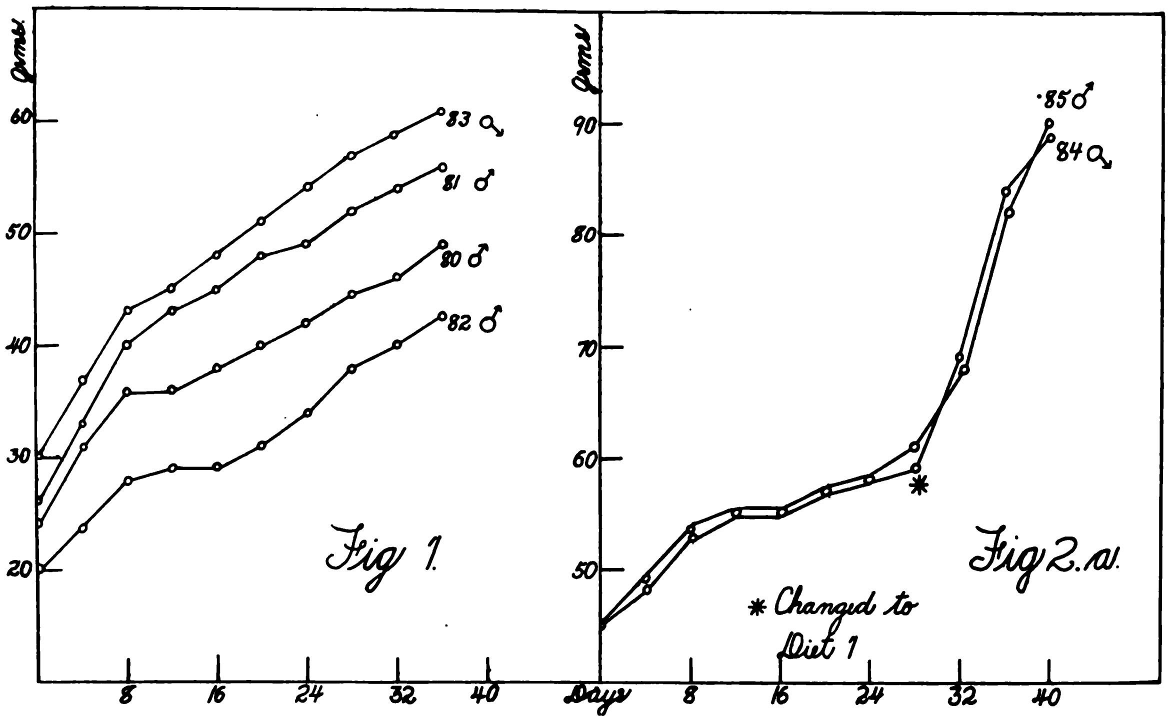

FIG. 1. Rats 80, 81, 82, and 83 were kept on a diet containing decomposed phosphotungstic acid precipitate from yeast.

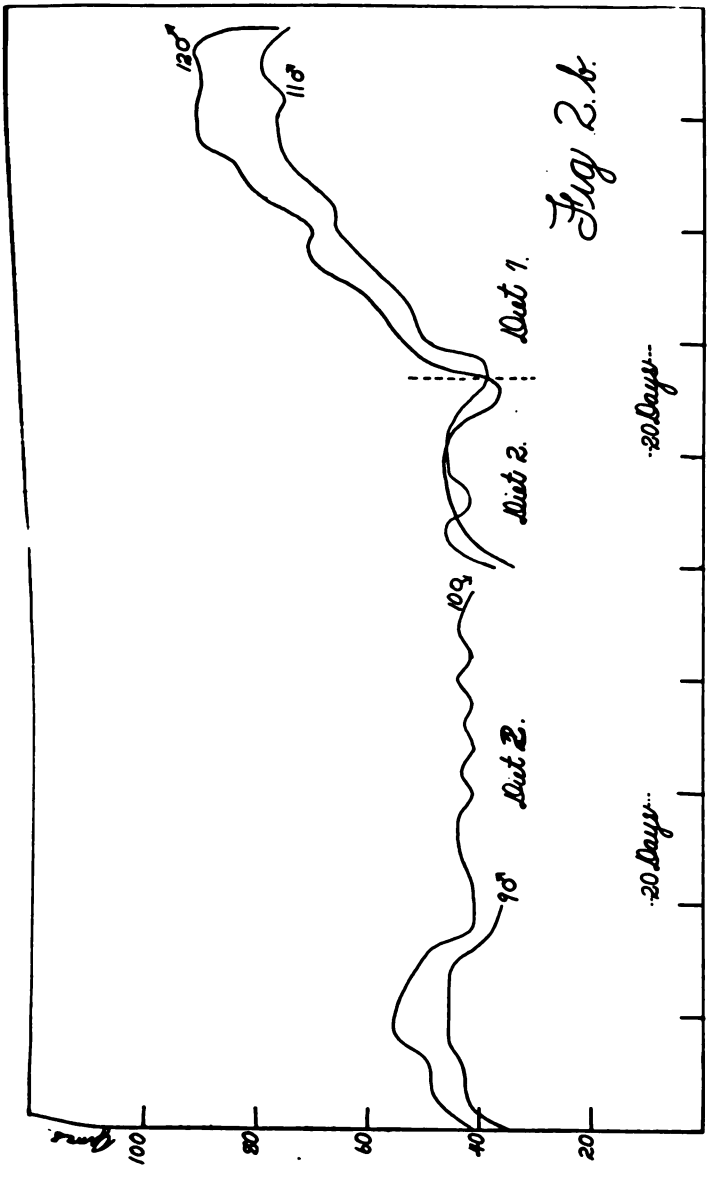

FIG. 2b. Rats 9 and 10 were kept on a diet containing phosphotungstic acid filtrate throughout the experiment. Rats 11 and 12 were changed to phosphotungstic precipitate at the point marked on the chart, with a marked improvement in growth and general appearance.

Rat 84. Female.
Rat 85. Male.
0–28 days Diet 2.
29–39 " " 1.

Days.	Weight.		Average daily food intake.
	84.	85.	
	gm.	*gm.*	*calories.*
0	45	45	
4	48	49	28.6
8	53	54.5	46.3
12	55	55	42.9
16	55	55	35.9
20	57	57	50.9
24	58	58	48.6
28	59	61	47.9
32	69	68	54.6
36	84	82	70.3
39	89	90	66.3

SUMMARY.

The fractionation of yeast with phosphotungstic acid shows that the growth-promoting substance is carried down with the precipitate and a large part of its activity is lost during the fractionation. The instability of this substance when fractionated with silver salts presents greater difficulty than that experienced during the fractionation of the beri-beri vitamine. It seems possible that both these problems can only be solved when more adequate methods are available.

THE REACTION BETWEEN AMINO-ACIDS AND CARBOHYDRATES AS A PROBABLE CAUSE OF HUMIN FORMATION.*

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The study of the black substances obtained when proteins are hydrolyzed in strong acid solution is of great interest at the present time on account of their bearing on the natural melanins and on the quantitative determination of certain amino-acids in proteins. Grindley[1] and his coworkers state that humin nitrogen causes an error in the analysis for amino-acids of common foodstuffs when the Van Slyke amino nitrogen determination is directly applied to them. This view on theoretical grounds was also expressed by Hart and Bentley.[2] It is therefore very important to know more about the structure and mode of formation of these compounds.

Mulder[3] was the first to show that albumins separate flocculi of a brown or black color on being boiled with concentrated hydrochloric or sulfuric acids. Hausmann[4] made similar observations with globin. Samuelly[5] pointed out that the formation of these "artificial melanins" or "melanoidins" might be a secondary reaction between amino-acids and carbohydrates. Maillard[6] conducted experiments along this line and found a number of them reacted with sugars. His experiments, however, we carried on in aqueous solutions at a very high concentration and te perature and it is doubtful whether under these conditions the reacti is similar to what takes place in the formation of either the "natur or artificial melanins. It will be shown later in this paper that not the amino-acids found reactive by Maillard reacted at all at low co centration in water. Gortner and Blish[7] made the important discove that when tryptophane is boiled with sugar in 22.9 per cent hydrochlo acid solution 86 per cent of its nitrogen is converted into humin nitrog They conclude from their experiments that tryptophane alone is respon ble for humin formation. Grindley and his coworkers[1] disagree with t conclusion since they found evidence that other amino-acids give the sa reaction.

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* The work described in this article forms part of a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Wisconsin.

[1] Grindley, H. S., and Slater, M. E., *J. Am. Chem. Soc.*, 1915, xxxvii, 2762.

[2] Hart, E. B., and Bentley, W. H., *J. Biol. Chem:*, 1915, xxii, 477.

[3] Mulder, G. J., in Mann, G., Chemistry of the Proteids, London, 1906, 87.

[4] Hausmann, W., *Z. physiol. Chem.*, 1900, xxix, 140.

[5] Samuelly, F., *Beitr. chem. Phys. u. Path.*, 1902, ii, 355.

[6] Maillard, L. C., *Compt. rend. Acad.*, 1912, cliv, 66.

In view of these conflicting statements and in the hope that t study of the reaction between amino-acids and carbohydrat would throw some light on the structure and mode of formati of the humin substances, it was thought worth while to determi (1) Which amino-acids react with carbohydrates under a giv set of conditions. (2) Whether different sugars behave ali toward the same reactive amino-acid. (3) What group of t reactive amino-acids takes part in the reaction.

REVIEW OF THE LITERATURE.

Udránszky[8] and Hoppe-Seyler[9] have shown that when sugar is boil with acids humin substances are formed, and if boiled in the presence a nitrogenous material, the humins may also contain nitrogen. Udrá zky found, for example, that glucose and urea boiled together in stro hydrochloric acid solution formed humins which contained about 6 per cent nitrogen.

Samuelly[6] was the first to study the behavior of humins, or "melan dins," towards oxidizing and reducing agents. He prepared his "melan dins" from commercial serum albumin according to Schmiedeberg' method modified by himself. He subjected his product to the action

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[7] Gortner, R. A., and Blish, M. J., *J. Am. Chem. Soc.*, 1915, xxxvii, 16 After this work was completed and sent for publication another arti by Gortner on humin formation appeared (*J. Biol. Chem.*, 1916, xx 177). In this article Gortner admits that amino-acids other than tryp phane may be involved in humin formation, which is in harmony wi the results reported here.

[8] Udránszky, L. v., *Z. physiol. Chem.*, 1888, xii, 33.

[9] Hoppe-Seyler, F., *Z. physiol. Chem.*, 1889, xiii, 66.

[10] Schmiedeberg, O., *Arch. exp. Path. u. Pharm.*, 1897, xxxix, 1.

HI and PI_3 in a closed tube kept for 8 hours at 200–210°C. Among other products he obtained pyrrol, as detected by the color reaction with pine shavings, and either pyridine or piperidine or some derivative of either one of these bases. Ammonia was also evolved in large amounts. None of the reduction products obtained as above gave indol or skatol on fusion with alkalies, while the humins before reduction gave on similar treatment an unmistakable odor of both. Samuelly also tried reduction with zinc dust in a current of hydrogen. From this treatment he obtained pyridine, pyrrol-like substances, skatol, and small amounts of an aromatic compound of the benzaldehyde series. The same investigator prepared humin substances from some amino-acids and glucose. He[11] heated for 18 hours a mixture of 10 gm. of glucose, 50 cc. water, 15 cc. concentrated HCl (sp. gr. 1.19), and sufficient amount of the different amino-acids so as to have in the solution 0.7 gm. of nitrogen. He tried ammonium chloride, urea, acetamide, glycocoll, aspartic acid, cystine, and tyrosine. In each case he found nitrogen in the melanin. No attempt was made, however, to determine whether the humin nitrogen formation was due to adsorption or to a definite reaction. It is interesting to note that all of the humins so prepared gave off pyrrol on dry distillation with zinc dust, no pyridine, and on fusion with alkalies only the humin prepared from tyrosine produced an odor of indol. Nencki[12] and Berdez[13] obtained similar results with the natural melanins. By alkali fusion these authors obtained from tumor melanin, indol, skatol, volatile fatty acids, hydrocyanic acid, succinic acid, and other unidentified products. Pyrrol was obtained on dry distillation, and after heating the melanin to 300°C. for some time, upon addition of an alkali pyridine was detected.

Gortner and Blish[7] heated pure zein, plus tryptophane, plus carbohydrate in 22.86 per cent HCl, and obtained 16.5 per cent of the total nitrogen of the mixture in the humin form. When tryptophane alone was heated with sugar in acid solution 86.56 per cent of its nitrogen was found in the humins. On the other hand, when histidine plus zein was heated with acid only 0.51 per cent of the total nitrogen was found in the humins. This amount was almost the same as that obtained when zein alone was heated in acid. They did not try, however, heating zein, plus histidine, plus sugar, in acid. Among the conclusions these authors draw from their experiments are the following: (1) The humin nitrogen belongs to "no amino-acids other than tryptophane." (2) "The reaction involved . . . . is probably the condensation of an aldehyde with the —NH group of the tryptophane nucleus." (3) Histidine can be eliminated "as a factor in the formation of humin nitrogen."

Grindley and Slater[1] have tried to apply the Van Slyke amino nitrogen determination directly to the analysis of feedingstuffs. As is to be expected, on account of the high carbohydrate content of these, the humin fracti in their nitrogen distribution is very high, varying from 3.85 per cent blood meal to 15.79 per cent in alfalfa hay, expressed as *per cent* of the tot nitrogen. In discussing the origin of these humin substances these inves gators disagree with the conclusion arrived at by Gortner and Blish, th the humin nitrogen of protein hydrolysis has its origin exclusively in t tryptophane nucleus, since they have obtained "results that clearly ind cate that in addition to tryptophane a number of other amino-acids wh gently boiled with 20 per cent HCl for 24 to 30 hours in the presence pure glucose give humin nitrogen. Preliminary experiments show th under the above treatment 4.7 to 6.3 per cent of the total nitrogen of lysi and cystine respectively is separated as humin nitrogen."

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[11] Samuelly, *Beitr. chem. Phys. u. Path.*, 1902, ii, 383.

[12] Nencki, M., and Sieber, N., *Arch. exp. Path. u. Pharm.*, 1887, xxiv, 17.

[13] Berdez, J., and Nencki, M., *Arch. exp. Path. u. Pharm.*, 1886, xx, 346.

Since Bourquelot and Bertrand discovered tyrosinase, this enzyme h received much attention from a great number of investigators. On that part of the work relating to the action of tyrosinase on the differe amino-acids and related substances will be reviewed here.[14]

The effect of tyrosinase on tyrosine is described by Bertrand.[15] A sol tion of tyrosine to which an extract of tyrosinase is added first becom red, then inky black, and finally deposits a black precipitate. He prov definitely that atmospheric oxygen is essential to the change by conduc ing experiments *in vacuo* and in the air. Von Fürth and Schneider[16] us the blood (hemolymph) of the pupæ of a butterfly, *Deiciphila elpen* They separated the enzyme from the other substances in the blood by fra tional precipitation with ammonium sulfate. It was found to give a ye lowish red substance with pyrochatechol; with hydroquinone it gave red solution, which then became turbid and finally deposited a conside able brownish precipitate. It also acted on adrenalin, giving a dirty brow color. Oxyphenylethylamine became yellowish brown and finally ga an olive-colored precipitate. But tyrosinase has no action on case itself. The same authors isolated the black substance produced from t rosine by the tyrosinase of *Deiciphila* pupæ and determined its eleme tary composition. Below is given a comparison between the percenta composition of this black substance and of tyrosine respectively:

	Black substance (humin) from tyrosine. *per cent*	Tyrosine. *per cent*
C	55.66	59.60
H	4.45	6.08
N	13.74	7.74
O	26.37	26.58

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[14] Bourquelot and Bertrand, G., *Bull. Soc. Mycol.*, 1896, xii, 18. A co plete list of references up to the time of its publication is found in Kastle Oxidases, *Bull. Hyg. Lab.*, 59.

[15] Bertrand, G., *Compt. rend. Soc. biol.*, 1896, cxxii, 1215.

[16] Von Fürth, O., and Schneider, H., *Beitr. chem. Phys. u. Path.*, 190 i, 229.

In the formation of this "artificial melanin" from tyrosine there is an increase in the nitrogen content from 7.74 to 13.74. Such an increase is only conceivable in one of two ways; either there is a breaking up of the tyrosine molecule, or some other nitrogenous substance besides tyrosine takes part in the formation of the melanin. The latter must be the case since tyrosinase, being but a weak oxidizing agent, would be unable to break down the benzene nucleus. The nitrogenous compound that took part in the reaction must evidently have come from the tyrosinase preparation itself. This black product also yields a skatol-like odor on fusion with alkali. In connection with the wide distribution of tyrosinase in both the vegetable and animal kingdom the following is quoted from Kastle's monograph:

"Von Fürth and Schneider are therefore of the opinion that probably wherever melanotic pigments occur in the living tissues of the lower and higher animals they originate as the result of the action of appropriate enzymes on substances of aromatic nature. They point out in this connection that Salkowski and Jacoby have shown independently that tyrosine results from the autolysis of various animal tissues. It would seem likely, therefore, that in the formation of melanotic pigments two ferments are jointly concerned: one, an autolytic ferment capable of splitting off tyrosine or a similar aromatic complex from the protein molecule, and the other tyrosinase, which transforms the tyrosine into melanin."

But one of the most interesting phases of the investigations on tyrosinase is that relating to its effect on the products of protein degradation and related substances. Bertrand and Rosenblatt[17] have found that this enzyme acts equally well upon racemic and *l*-tyrosine. Chodat and Staub[18] discovered that albumoses do not give a red color with tyrosinase but glycyltyrosine anhydride gives such a coloration. In a later article[17] these authors observed that glycine, leucine, and alanine retard the action of tyrosinase; that dipeptides such as tyrosine anhydride, and glycyltyrosine anhydride produce yellow substances which do not become black as does tyrosine itself. When, however, glycine, leucine, or alanine is present, a red coloration similar to that resulting from tyrosine is obtained: glycyltyrosine anhydride with glycine gives a rose color changing to bluish green; with alanine the color is deeper red, with leucine deep brown. But their most striking discovery is that phenylalanine is not acted on by tyrosinase. This, however, acts readily on *p*-cresol, less readily on *m*-cresol, and still less readily on *o*-cresol. In fact these same authors observed that the enzyme acts most readily on the para-homologues of phenol. Amino-acids like glycine increase the rapidity of the action of tyrosinase on *p*-cresol, producing a violet color which ultimately becomes blue. Bertrand undertook to investigate the action of tyrosinase from wheat bran on compounds analogous to tyrosine and to phenylalanine; that is, compounds with and without the phenolic hydroxyl group. Thus he found phenylalanine, phenylethylamine, phenylmethylamine, phenylaminoacetic acid, phenylpropionic acid, phenylacetic acid, alanine, and glycocoll produced no coloration at all. On the other hand the following compounds with phenolic hydroxyl groups produced coloration as follows:

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[17] Bertrand, G., and Rosenblatt, M., *Compt. rend. Soc. biol.*, 1908, cxlvi, 304.

[18] Chodat, R., and Staub, *Arch. Sc. Phys. Nat.*, 1907, xxiii, 265; xxiv, 172.

Tyrosine	Grenadine-red, inky black.
p-Hydroxyphenylethylamine	Grenadine-red, olive-black.
p-Hydroxyphenylmethylamine	Orange-yellow, orange-red, clear maroon.
p-Hydroxyphenylamine	Orange, mahogany-red, brown.
p-Hydroxyphenylpropionic acid	Orange-yellow, grenadine-red, brown.
p-Hydroxybenzoic acid	Rose, orange, yellow.
p-Cresol	Yellow, orange, red.
Phenol	Yellow, orange, red, brown.

He concludes, therefore, that tyrosinase acts only on those compounds containing a phenolic group.

In 1907 Abderhalden and Guggenheim[19] published an interesting article on the effect of tyrosinase from *Russula delica* on tyrosine, tyrosine-containing polypeptides and other related substances. They observed that glycocoll, *d*-alanine, *d*-valine, *l*-proline, *d*-serine, *d*-*l*-isoserine, and *l*-phenylalanine retard the action of tyrosinase on tyrosine only slightly unless present in very large concentrations. The largest concentration used was molar; *l*-aspartic acid and *d*-glutamic acid, however, even when present at a concentration of 0.01 molar retard the action considerably. The same authors found that the enzyme has no effect on diiodotyrosine, *l*-phenylalanine, *l*-proline, or cystine. But *l*- and *d*-tyrosine, homogentisic acid, and tryptophane showed a color change. Particularly interesting was the case of *d*-tryptophane. The authors state that at first they thought that the coloration with tryptophane may be due to traces of tyrosine. They, however, used a very pure product. They repeated their experiment but always came to the same result. Furthermore, they tried the effect of tyrosinase on solutions of tryptophane-containing polypeptides and found development of color. They therefore conclude that this coloration must not be ascribed to the presence of traces of tyrosine. Still more interesting is the fact that neither indol nor skatol were found to produce coloration. Abderhalden and Guggenheim in the same article describe the effect of tyrosinase on polypeptides containing tyrosine. The color developed in these cases is modified to some extent by the nature of the amino-acid combined with the tyrosine in the polypeptide. Addition of some amino-acids were also found either to accelerate or to retard the action of tyrosinase on the polypeptide. Thus proline accelerates considerably the action of the oxidase on glycyl-*l*-tyrosine anhydride, while aspartic acid and glutaminic acid retard the action. On the other hand halogen derivatives of the polypeptides were not acted upon by tyrosinase. The same authors also found, as did Bertrand, that tyrosinase acts on phenol, giving a brown color, which was modified by amino-acids. Thus glycocoll plus phenol gave a cochineal color, while proline and phenol gave violet. The authors finally concluded that the amino-acids, when present, apparently take part in the production of the pigment. In a later article[29] these same authors point out that tyrosinase acts on adrenalin with the rapid production of a red color and ultimately dark red flocculi. All three isomers of adrenalin are affected with equal rapidity.

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[19] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1907–08, liv, 331.

It is to be regretted that in none of the above cited contributions was either arginine, histidine, or lysine tried. It is hoped that this omission will be filled in the near future.

EXPERIMENTAL.

The fact that zein, when boiled with glucose in 22.68 per cent hydrochloric acid solution, increases its humin nitrogen from 0.56 to 1.84 per cent indicates, as Grindley and his coworkers[1] suggested, that other amino-acids besides tryptophane take part in nitrogenous humin formation. Only a small per cent of some of these amino-acids may take part in this formation so that only by working with the individual amino-acids is it possible to determine whether the humin nitrogen was due to a definite reaction or to an adsorption. Again it is only by working under approximately the same set of conditions that it is possible to detect differences in behavior between the different amino-acids. The following procedure was, therefore, adhered to as consistently as practicable.

The amino-acid, plus sugar, plus 50 cc. of water or hydrochloric acid solution of the specified strength was heated for 48 hours in a 300 cc. Kjeldahl flask on a sand bath. The flask was provided with a reflux condenser made from a large test-tube fitted with cork and tubings for a current of cold water. After heating, the digestion mixture was neutralized with the calculated amount of sodium hydroxide solution. The salt thus formed coagulated most of the precipitate that may have existed in a colloidal state in the solution. The mixture was then filtered into 200 cc. graduated flasks and the humin was washed repeatedly with boiling water until the flask was filled to the mark. Th amount of washing was found to be sufficient to remove almo all of the adsorbed amino-acid which could be removed by th treatment alone. The humin with the filter paper was th Kjeldahled. The filtrate was either Kjeldahled or Van-Slyk or used for both determinations. 25 cc. portions were taken f the Kjeldahl and 10 cc. portions for the Van Slyke determinatio

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[29] Abderhalden and Guggenheim, *Z. physiol. Chem.*, 1908, lvii, 329.

The nitrogen content of the amino-acids was determined eith by Kjeldahl's or by Van Slyke's method or by both. The *p* *cent* of nitrogen was the only thing used to establish the identi and purity of the compounds.

The following amino-acids were furnished by Professor Ha

Amino-acid.	Nitrogen. Found. *per cent*	 Theoretical *per cent*
Alanine	15.70	15.75
Cystine	11.2–11.6	11.67
Tyrosine	7.67	7.72
Lysine hydrobromide ($2C_6H_{14}N_4O_2 . HBr . H_2O$)	14.72	14.32
Tryptophane	6.44 (Amino N)	6.86
Phenylalanine*	6.95	6.94

* The phenylalanine was kindly furnished by Dr. T. B. Osborne New Haven.

The following amino-acids were prepared:

Amino-acid.	Nitrogen. Found. *per cent*	 Theoretical *per cent*
Leucine, from zein	10.90	10.70
Proline, from zein, also from gelatin	12.20	12.17
	(No amino N)	
Glutaminic acid, from gliadin	7.17	7.64
Arginine (free) from gelatin	29.95	32.20
Amino N	7.70	8.04
Histidine dihydrochloride, from blood	17.25	18.42
Amino N	5.76	6.14

In the preparation of the above amino-acids the directio given in Abderhalden's *Arbeitsmethoden* were followed. Th *per cents* of nitrogen found for arginine and histidine respective were not quite up to the theoretical, but since the amino nitrog was almost one-fourth of the total in the arginine sample and on third in the histidine, it was evident that the samples of bot these amino-acids were free from other amino-acids, their low total nitrogen content being due to moisture. The nitrogen determinations of these amino-acids were made on the same day that the experiments on humin formation were started.

Due to the scarcity of material it was found impossible to recrystallize some of the amino-acids in order to obtain as pure a product as could be desired.

The results are shown in the following table. All the experiments were in duplicate and average figures are given.

The results show that neither alanine nor leucine give humin nitrogen. Glutaminic acid when boiled with sugar in 2 per cent acid solution yields some humin nitrogen, but none in 20 per cent acid. Attention is called to the fact that glutaminic acid on heating even at the concentration used seems to form pyrrolidon carboxylic acid readily, as evidenced by the loss of activity of its amino nitrogen in Experiments 13, 14, and 16. Such a formation does not take place in strong acid. Phenylalanine yields about 1.65 per cent of its nitrogen in the humin in 20 per cent acid solution. Proline does not give humin nitrogen with glucose with 20 per cent acid, but seems to react to some extent with xylose and fructose in 4.15 per cent acid solution. Cystine with 20 per cent HCl yields about 3.1 per cent humin nitrogen and the noteworthy fact about this amino-acid is the deeply colored filtrate it produced. The same was observed with the filtrate from the tyrosine-sugar experiments. As much as 15 per cent of tyrosine nitrogen may be converted into humin nitrogen.

The cases of the three hexone bases are particularly interesting. When boiled with sugars in 20 per cent HCl solution all three yield some of their nitrogen as humin nitrogen. Arginine and lysine, with sugar, give more deeply colored filtrates than histidine. If the deep coloration of the filtrate indicates reaction, then it must be stated definitely that in the case of cystine, tyrosine, arginine, and lysine in 20 per cent HCl, the humin nitrogen is due to a reaction and not to an adsorption. Another fact that supports the contention that a definite reaction is responsible for humin formation at least in the case of tyrosine is that phenylalanine gives but little humin nitrogen. If this were a case of adsorption, then there should probably be no differ-

TABLE I.

Record of Results.

		1	2	3	4	5	6	7	
Experiment No.	Treatment.	Humin N (Kjeldahl).	Total N in filtrate (Kjeldahl).	Amino N in filtrate (Van Slyke).	Calculated amino N in filtrate.	Amino N difference.	Total N found (column 1 + column 2).	Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Alanine.								
1	0.089 gm. + 20 per cent HCl.......	0.0		13.88	13.98	0.10		13.98	Filtrate colorless.
2	0.089 gm. + 0.720 glucose + 20 per cent HCl........................	0.0		13.81	13.98	0.17		13.98	" light yellow.
3	0.089 gm. + 0.600 gm. xylose + 20 per cent HCl*								
4	0.089 gm. + 0.720 gm. fructose + 20 per cent HCl....................	0.0		14.00	13.98	−0.02		13.98	" " "
5	0.089 gm. + 2.0 gm. glucose + 20 per cent HCl....................	0.0		14.01	13.98	−0.03		13.98	" " "
6	0.0415 gm. + 0.720 gm. glucose + 20 per cent HCl................	0.0		6.51	6.55	0.04		6.55	" " "
	Leucine.								
7	0.131 gm. + 4.15 per cent HCl......	0.0		14.36	14.36	0.0		14.36	" colorless.
8	0.131 gm. + 0.720 gm. glucose + 4.15 per cent HCl.................	0.0		14.33	14.36	0.03		14.36	" light yellow.
9	0.131 gm. + 0.720 gm. fructose + 4.15 per cent HCl.................	0.0		14.30	14.36	0.06		14.36	" " "

* Determination lost.

10	0.131 gm. + 0.720 gm. glucose + water	0.0		14.40	14.36	−0.04		14.36	Filtrate light yellow.
11	0.131 gm. + 0.720 gm. glucose + 20 per cent HCl	0.0		14.40	14.36	−0.04		14.36	" " "
	Glutaminic acid.								
12	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	0.10 0.35 per cent		28.60	28.60	0.00		28.70	" " "
13	0.400 gm. + 2 gm. glucose + 20 per cent HCl	0.46 1.65 per cent		22.00	28.24	6.70		28.70	" " "
14	0.162 gm. + 0.720 gm. glucose + water	0.05 0.03 per cent		4.47	11.55	7.13		11.60	" colorless.
15	0.162 gm. + 0.720 gm. glucose + 20 per cent HCl	0.00		11.80	11.60	−0.20		11.60	" light yellow.
16	0.162 gm. + water	0.00		4.58	11.60	7.02		11.60	" colorless.
	Phenylalanine.								
17	0.4 gm. + 2.0 gm. glucose + 20 per cent HCl	0.46 1.65 per cent		27.50	27.32	−0.18		27.80	" light yellow.
18	0.4 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00		28.00	27.80	−0.20		27.80	" colorless.
19	0.2015 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00		28.00	27.80	−0.20		27.80	" "

TABLE I—*Continued.*

Experiment No.	Treatment.	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Proline.								
20	0.115 gm. + 4.15 per cent HCl	0.00	14.07				14.07 100.1 per cent	14.00	Filtrate almost colorless.
21	0.115 gm. + 0.720 gm. glucose + 4.15 per cent HCl	0.00	14.12				14.12 100.9 per cent	14.00	" light yellow.
22	0.115 gm. + 0.600 gm. xylose + 4.15 per cent HCl	0.53 3.66 per cent	13.10				13.63 97.5 per cent	14.00	" " "
23	0.115 gm. + 0.720 gm. fructose + 4.15 per cent HCl	0.33 2.36 per cent	13.98				14.31 102.0 per cent	14.00	" " "

25	0.0010 gm. + 2.0 gm. glucose + 20 per cent HCl	0.00							Filtrate light yellow.
	Cystine.								
26	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.25 2.9 per cent							Filtrate yellowish brown.
27	0.400 gm. + 2.0 gm. glucose + 1 per cent HCl	0.00							Filtrate almost colorless.
28	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.36 3.1 per cent	43.20	43.60	43.44	−0.16	44.56 99.4 per cent	44.8	Filtrate yellowish brown.
29	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	1.36 3.1 per cent	43.12	43.60	43.44	−0.16	44.48 99.3 per cent	44.8	Filtrate yellowish brown.
30	0.200 gm. + 2.0 gm. glucose + 0.1 cent HCl	0.00	20.84				20.84 89.5 per cent	23.3	Filtrate light yellow.
31	0.200 gm. + 2.0 gm. proline + 2.0 gm. glucose + 20 per cent HCl	1.61*	45.70				47.31 99.4 per cent	47.70	Filtrate yellowish brown.

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*3.38 per cent of total N or 6.9 per cent of cystine N.

TABLE I—*Continued*.

Experiment No.	Treatment.	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino-N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
	Tyrosine.								
32	0.1586 gm. + 20 per cent HCl........	0.00		12.22	12.22			12.22	Filtrate brownish.
33	0.1586 gm. + 0.720 gm. glucose + 2.5 per cent HCl..................	0.20		12.01	12.02	0.01		12.22	" yellowish brown.
34	0.1586 gm. + 0.720 gm. glucose + 20 per cent HCl..................	0.84		11.40	11.38	−0.02		12.22	" brownish.
35	0.1586 gm. + 2.0 gm. glucose + 20 per cent HCl....................	1.33		10.62	10.89	0.27		12.22	" "
36	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl....................	4.64		26.00	26.06	0.06		30.70	" "
	Arginine.								
37	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl....................	2.34 1.97 per cent	115.00	28.20	28.90	0.70	117.34 98.4 per cent	119.5	" yellowish brown.
38	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl....................	2.77 2.33 per cent	115.50	28.70	28.95	0.25	118.27 99.0 per cent	119.5	" "
39	0.400 gm. + 2.0 gm. glucose + water.	0.35 0.29 per cent	115.00	21.70	28.90	7.20	115.35	119.5	" brownish car-

	Lysine.								
40	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.54 2.62 per cent	57.5	57.50	57.50	0.00	58.94 100.5 per cent	58.90	Filtrate yellowish brown.
41	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	1.47 2.50 per cent	57.4	57.00	57.40	0.40	58.77 99.9 per cent	58.90	" " "
42	0.2 gm. + 1.0 gm. glucose + water.	0.28 0.48 per cent	28.3	23.70	28.60	4.90	28.58 98.0 per cent	29.20	" carmine.
	Histidine.								
43	0.1915 gm. + 20 per cent HCl			14.00	14.00			42.00	" light yellow.
44	0.1915 gm. + 0.360 gm. glucose + 4.15 per cent HCl	0.61 1.45 per cent		12.19	13.80	1.61		42.00	" " "
45	0.1915 gm. + 0.720 gm. glucose + 20 per cent HCl	0.77 1.84 per cent		12.81	13.74	0.93		42.00	" " "
46	0.1915 gm. + 0.600 gm. xylose + 4.15 per cent HCl	1.08 2.58 per cent		12.38	13.62	1.24		42.00	" " "

TABLE I—*Concluded.*

Experiment No.	Treatment.	1 Humin N (Kjeldahl).	2 Total N in filtrate (Kjeldahl).	3 Amino N in filtrate (Van Slyke).	4 Calculated amino N in filtrate.	5 Amino N difference.	6 Total N found (column 1 + column 2).	7 Total N calculated.	Remarks.
		mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
47	0.1915 gm. + 0.600 gm. xylose + water	0.68 1.62 per cent		11.53	13.80	2.27		42.0	Filtrate yellowish brown.
48	0.400 gm. + 2.0 gm. glucose + 20 per cent HCl	0.80 1.16 per cent	68.00	23.20	22.70	−0.50	68.80 99.8 per cent	69.0	" light yellow.
49	0.400 gm. + 2.0 gm. fructose + 20 per cent HCl	0.77 1.12 per cent	67.20	23.40	22.40	−1.00	67.90 98.4 per cent	69.0	" " "
50	0.200 gm. + 1.0 gm. glucose + water	0.00	35.40	11.68	11.80	0.12	35.40 100.0 per cent	35.4	" " "
51	0.200 gm. + 1.0 gm. fructose + water	0.21 0.32 per cent	33.30	8.70	11.10	2.30	33.51 94.6 per cent	35.4	" deep brown.
	Tryptophane.								
52	0.1514 gm. + 1.0 gm. glucose + 20 per cent HCl	13.82	5.44	2.73	2.72	−0.01	19.26	19.50	" almost colorless.

ence in behavior between tyrosine and phenylalanine. In aqueous or very weak acid solution arginine, histidine, and lysine evidently react with sugar as indicated by the highly colored solutions produced and by the loss of activity of a large fraction of their amino nitrogen. Thus, when arginine plus glucose was boiled in water there was a very deep coloration of the solution (Experiment 39), and at least 25 per cent of the amino nitrogen became inactive towards nitrous acid. Lysine behaved similarly (Experiment 42), 17 per cent of the amino nitrogen becoming inactive towards nitrous acid. Histidine acted likewise (Experiments 47 and 51), 16.2 per cent of its amino nitrogen becoming inactive. These facts show that in the cases of histidine and arginine the α-amino nitrogen takes part in the reaction. In the case of lysine it is difficult to establish which amino group is reactive, since at the time the amino nitrogen in the filtrate was determined the temperature in the laboratory was about 35°C. and at this temperature it was found that both the α- and the ϵ-amino group of lysine react with nitrous acid in 5 minutes, as may be seen in the amino nitrogen determination of the filtrate (Experiments 40 to 42). It is to be noted that in these cases some loss of nitrogen also took place. It may be that during the reaction some ammonia was given off.

The result with tryptophane is in agreement with the work of Gortner and Blish[7] in that a greater portion of the tryptophane nitrogen is converted into humin. The strength of the acid used here and the different procedure followed may account for the difference in the *per cent* of tryptophane nitrogen found in the humin which according to the above named authors was 86 per cent while in these experiments only about 71 per cent was obtained. Due to a lack of material it was impossible to repeat the experiment with tryptophane.

In order to determine which atomic groupings in tyrosine, cystine, and tryptophane were responsible for humin formation, the humin from each one of these amino-acids was dissolved in 0.1 N alkali and Van-Slyked. It was believed that if the amino groups in this humin remained intact they should still give the nitrous acid reaction. The results are as follows:

	Humin nitrogen. mg.	Reactive with HNO_2. mg.
Tyrosine	2.360	2.45
Cystine	0.974	0.88
Tryptophane	13.820	1.90

From these results it must be concluded that in the case of tyrosine and cystine it was not the amino group that reacted with sugar to form humin but some other group, probably the (OH) in tyrosine and the (S-S) in the case of the cystine. If this were the case, then the cystine would presumably undergo reduction before reacting with the sugar.

In order to determine whether, as Gortner and Blish suggested, the furfurol obtained from sugar was responsible for the reaction, Experiments 54, 55, and 56 were performed as follows:

Experiment No.		Humin N. mg.	Per cent of total N.
54	0.2 gm. cystine + 2 cc. furfurol + 20 per cent HCl	7.00	32.0
55	0.2 gm. tyrosine + 2 cc. furfurol + 20 per cent HCl	8.40	55.0
56	0.2 gm. arginine + 2 cc. furfurol + 20 per cent HCl	12.75	21.5

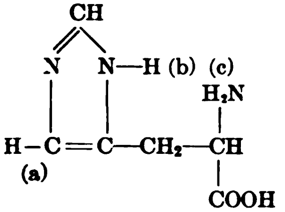
These results tend to show that the furfurol formed from sugars under the influence of acids may to a great extent be responsible for humin formation.

As to the effect of the different sugars on the reactive amino-acid, Experiments 20, 21, 22, 29, 38, and 46 show that xylose and fructose give higher results than glucose as a rule. This is to be expected, if it is admitted that furfurol or some other simple aldehyde is the active substance in these reactions.

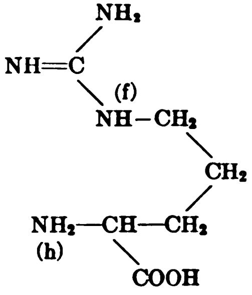
DISCUSSION OF RESULTS.

Some evidence is given which shows that the α-amino groups of arginine, histidine, and tryptophane take part in the reaction with sugars. On the other hand, the α-amino groups of alanine and leucine are unable to give the same reaction. Glutaminic acid and phenylalanine, although giving some humin nitrogen, likewise furnish no indication of reaction. At least for the present it may be admitted that the humin nitrogen in these cases—

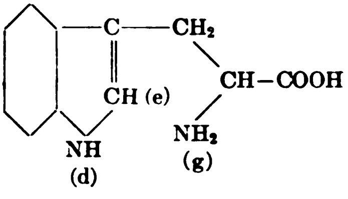
glutaminic acid and phenylalanine—was due to adsorption and not to a reaction. It was also shown that in tyrosine the reactive group is presumably the (OH) and surely not the α-NH_2. In cystine, as shown above, the α-amino group remained intact, so that presumably the reaction was with the mercaptan group. The question may then be asked: Why are the α-NH_2 groups of arginine, histidine and tryptophane more reactive than those of the other amino-acids? An attempt to explain this difference in behavior of the amino-acids towards carbohydrates, based on the present work and on some of the contributions reviewed in the first part of this paper, is here offered. It is generally stated that the properties of a compound are functions of its structure. It is, therefore, to the structure of these amino-acids that we must look for an explanation of their different behavior towards carbohydrates. The structural formulas of histidine, tryptophane, and arginine are given below.



Histidine.



Arginine.

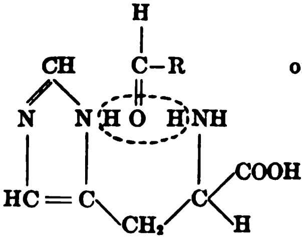


Tryptophane.

Several investigators have advanced the idea that humin formation is dependent on the presence of labile hydrogen[*] in the amino-acid molecule (Samuelly, Grindley and Slater, etc.). Evidently judging from the results of the present work the two hydrogens of the α-amino groups of alanine and leucine are not labile enough to give condensation products with carbohydrates at least under the conditions of these experiments.

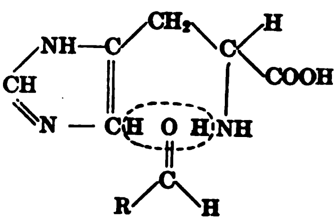
In histidine, arginine, and tryptophane, however, there are other labile hydrogens (a, b, c, d, e, f). The positions of these labile hydrogens with respect to the α-amino group are very favorable for ring formation. The reaction with a carbohydrate or furfurol may very well be thought of as taking place as follows:

Histidine.



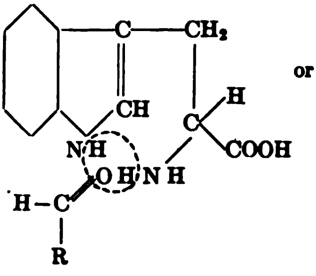
I

or



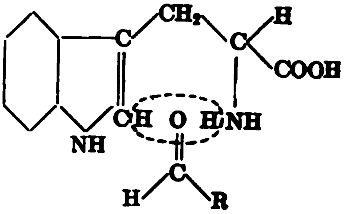
II

Tryptophane.



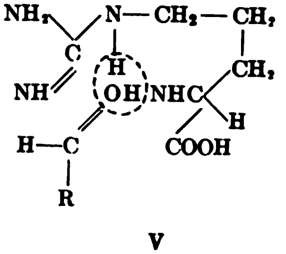
III

or



IV

Arginine.



V

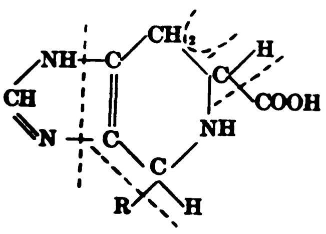
The following facts tend to support the idea of ring formation:

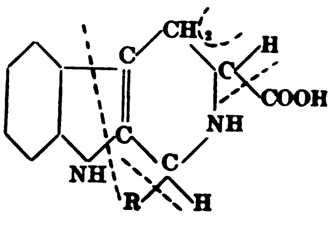
1. The intense color of the products.

2. Miss Homer[21] in her work on the condensation products of tryptophane with aldehydes, speaking of the action of glyoxal on this amino-acid, states:

> "Taking into consideration the necessity of the presence of an oxidizing agent and also the fact that the substance produced is intensely colored it is highly probable that in this reaction, besides the simple aldehyde condensation . . . . there has also been elimination of hydrogen accompanied by complex ring formation."

3. The fact that pyridine was obtained by Samuelly from his "melanoidins," was at one time used as an argument to indicate that a pyridine nucleus was found in proteins. This idea has been disposed of by Emil Fischer's work on proteins, but the fact remains that pyridine is found in the humin formed from proteins. This occurrence may be explained by Reactions II and IV thus:





4. The action of tyrosinase on tyrosine tends to support the idea of ring formation. Tyrosinase produces coloration with tryptophane but not with indol, skatol, or glycocoll. Therefore, the formation of the highly colored product requires the peculiar structure of tryptophane. This formation may be considered as taking place in the manner described above.

The differences in behavior between histidine, arginine, and tryptophane may again be referred back to the differences in their structure. Tryptophane being already a complex compound with a benzene and a pyrrol ring may form an insoluble four-ringed compound with furfurol, which is extremely resistant to the action of acid. This will explain why tryptophane is converted into humin almost quantitatively. On the other hand furfurol may form with histidine and arginine products which are still more or less soluble and in the presence of strong acid may be hydrolyzed back to the free amino-acids. Thus as in the case of glutaminic acid, no formation of a ring compound takes place in strong hydrochloric acid solution. This view will explain why in weak acid or in aqueous solution both histidine and arginine react more readily to form colored products than in strong acid solution.

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[21] Homer, A., *Biochem. J.*, 1913, vii, 111.

It is not claimed that the reaction given above gives the actual structure of the melanin molecule, since no evidence is available to indicate what happens to the rest of the molecule of the amino-acids during the reaction with sugars. This theory on humin formation is given here in the hope that it may serve as a guide for future work on the structure of these compounds.

No evidence was found in the present work to explain Samuelly's finding that when the humin obtained from sugar plus tyrosine was fused with alkalies, an odor of indol was obtained. It might have been possible that the tyrosine used contained traces of tryptophane which would explain the production of indol. Likewise the fact that pyrrol was obtained from his "melanoidins" can be traced back to the presence of the tryptophane nucleus in them.

Almost all of the experiments recorded in this paper were done with single amino-acids. There was found evidence (Experiment 31) to show that the reaction would be different, at least in the case of cystine and tyrosine, if other amino-acids were present in the reaction mixture with sugar. If cystine and proline were boiled together in the presence of glucose and 20 per cent HCl a larger amount of cystine nitrogen disappeared in humin formation than when cystine was boiled alone. The same was true when tyrosine and proline were boiled together. It would, therefore, be interesting to study the behavior of mixtures of different amino-acids when boiled with sugars, both in acid and aqueous solutions. Abderhalden and Guggenheim[19] working with tyrosinase along this same line already concluded that other amino-acids, when present, apparently take part in the production of the pigment.

CONCLUSIONS.

1. Alanine, leucine, phenylalanine, and glutaminic acid may be eliminated as important factors in humin formation, when subjected to the treatment used in these experiments. Proline, however, under certain conditions may be involved in humin formation.

2. The following amino-acids were responsible for humin formation, and in digestions, with 20 per cent HCl plus sugar, the proportion of their nitrogen disappearing was: Tyrosine, 15.0; cystine, 3.1; arginine, 2.33; lysine, 2.62; histidine, 1.84; tryptophane, 71.0 per cent.

3. Xylose and fructose were as a rule more reactive than glucose.

4. Arginine, histidine, and lysine reacted with sugars more readily in weak acid or aqueous, than in strong acid solutions.

5. Arginine, histidine, and tryptophane reacted with loss in reactivity of their amino nitrogen towards nitrous acid, but tyrosine and cystine reacted without any such loss.

6. A possible mode of reaction is suggested.

It is with pleasure that the writer acknowledges his obligation to Professor E. B. Hart, Chief of this Department, for giving him this problem, and for his many valuable suggestions during its execution.

THE DIRECT DETERMINATION OF UREA AND AMMONIA IN MUSCLE.

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(Received for publication, August 16, 1916.)

The determination of urea in blood and urine has been made both simple and accurate by the urease method of Marshall[1] as developed by Van Slyke and Cullen.[2] For some time the writer has had need of a suitable modification of this method in order to determine urea and ammonia in muscle and other tissue. Marshall[1] has successfully applied his urease method to the analysis of tissue, extracting with several portions of alcohol. The writer in collaboration with Fiske,[3] has used a similar method of extraction with alcohol, applying the method of Folin and Denis.[4] This procedure is apparently accurate but is tedious.

On account of the ease with which urea is capable of diffusing through cell membranes,[5] it appeared that the urease solution might be allowed to act directly upon the tissues containing urea, provided these tissues were ground finely, and enough time were allowed for the process of diffusion. At first the author tried grinding muscle with sand in a glass mortar, but gave up this procedure as unnecessary when it was found, a little later, that even coarsely chopped muscle tissue gave as high values for urea as samples that had been ground with sand to a very fine powder. That the urea quickly diffuses out of even rather coarsely chopped muscle was shown by the fact that samples allowed to stand in urease solutions for ½ hour gave, upon aeration, no lower figures for urea than did samples that had been allowed to stand with urease for 1 and 2 hour intervals. This fact makes it possible to determine urea in muscle directly, and the writer sees no reason why it may not be determined in the same manner in any other tissue.

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[1] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 493. Marshall, E. K., Jr., and Davis, D. M., *ibid.*, 1914, xviii, 53.

[2] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211; 1916, xxiv, 117.

[3] Fiske, C. H., and Sumner, J. B., *J. Biol. Chem.*, 1914, xviii, 288.

[4] Folin, O., *J. Biol. Chem.*, 1912, xi, 507.

[5] Gryns, G., *Arch. ges. Physiol.*, 1896, lxiii, 86, and others.

Van Slyke and Cullen have stated that the accuracy of the urease method is limited only by the error involved in titration, but I have found the greatest error to be in the process of aeration. The use of methyl red as indicator, of Jena glass, of sufficiently dilute acid and alkali solutions, of calibrated burettes, of a vertical plate glass mirror 2 feet back of the burettes as a means of avoiding parallax, and the precaution—as Van Slyke and Cullen advise—of keeping rubber stoppers and tubing from contact with strong alkalis and acids, make the process of titration an exceedingly accurate one.

EXPERIMENTAL PART.

Fresh muscle was obtained from cats and guinea pigs. They were etherized, killed by bleeding, and skinned; and the more accessible muscles removed with as little fat and fascia as possible. This muscle was minced in a "Universal" meat chopper. In order to obtain the tissue as homogeneous as possible, it was usually put through the meat chopper four or five times. The ground muscle was then transferred to a rubber-stoppered weighing bottle. A long glass spatula was found most useful in transferring samples of material to the bottoms of the large test-tubes (23 x 200 mm.) 9 cc. of water in portions were used to wash this spatula and to rinse down any bits of muscle that had stuck to the sides of the test-tubes. The most convenient weight of muscle to take as a sample was found to be from 2 to 3 gm. If larger amounts of muscle are used more water should be added to prevent the mass from becoming pasty. 1 cc. of a 5 per cent urease solution accurately made up was then added, the mixture well stirred with the end of the glass aeration tube, and after standing for ½ hour or longer the process of aeration was carried out. For the determination of ammonia in muscle the mixture was stirred and aerated at once.

As the ground muscle was always found to be acid, no acid phosphate was added to it; and there was no danger from loss of ammonia.

The urease used was made by the method of Van Slyke and Cullen,[3] with the modification that less water was employed in the extraction of the urease, and a larger volume of acetone was used to precipitate it. The ammonia content of this dry preparation was carefully determined. New urease solutions were made up each day, and kept on ice. The author found it convenient to weigh out the urease into a large glass mortar and add the desired amount of water from a pipette, grind, and then transfer to a flask.

With the exception of the urease, all the reagents used were purified until almost absolutely free from ammonia. The potassium carbonate was made ammonia-free by boiling its solution to dryness, and heating until anhydrous.

For the liberation of ammonia, 5 gm. of anhydrous potassium carbonate were added to the solution, in which there were always present 10 cc. of water. The acid used for absorbing the ammonia set free was 0.01 N, and the alkali used in running back, 0.0088 N. In order to increase the height of the absorbing column of acid, 10 cc. of water were always added; this made a height in the test-tube of 6 to 7 cm., and under the conditions of the experiments allowed no measurable amount of ammonia to escape. With alkali even as dilute as 0.0088 N it was found that by using methyl red as indicator, and a standard end-point of sodium acetate and acetic acid, titrations could be run to within a very small fraction of a drop of alkali. The acid and alkali solutions were never allowed to come into contact with any but Jena or "nonsol" glass, with exception of the burettes which were well seasoned; Jena glass absorption tubes were used. Both aeration and absorption tubes were provided with eight small holes, as Van Slyke and Cullen advise. One drop of caprylic alcohol was added to the test-tube containing the acid and fifteen drops to the aerated mixture. The time of aeration was usually 40 minutes with an air current of 2 to 3 liters per minute, always run slowly for the first 3 minutes.

It was found that when the speed of aeration was 5 liters per minute, as advised by Van Slyke and Cullen, it was necessary to keep the test-tubes containing the acid stoppered and to fit them with wide (8 mm.) bent tubes as traps to prevent loss of acid by spattering. Much more serious was the spattering of alkali from the test-tubes containing the aerated mixture. I found that no amount of caprylic alcohol would prevent the passing over into the acid of very slight amounts of fine spray. Although the alkali blown over with an air current of 5 liters per minute is very small in amount (usually 0.1 cc. of 0.01 N alkali with 20 minutes' aeration), this is not a negligible factor when 0.5 mg. or less of nitrogen is being dealt with. When the aeration is at a rate of not more than 3 liters per minute, this source of error is much lessened. The rate of aeration was measured by means of a Junkers gas meter and also by the method devised by Kober and Graves.[6]

Below are some results obtained by this method.

TABLE I.

Cat Muscle.

Weight.	Urease acted.	Mg. per 100 gm. moist muscle.	
		Urea and NH_3 N.	NH_3 N.
gm.	*hrs.*		
1.757	½	28.0*	
2.183	½	27.4*	
2.095	2	28.4	
1.811	2	28.6	
1.880	2	28.7	
1.177			10.4
1.486			10.0

* Once through chopper.

TABLE II.

Guinea Pig Muscle.

2.422	1	21.0	
2.476	1	20.6	
2.777	1	21.0	
2.760			14.6
3.165			14.1
2.521			15.3

[6] Kober, P. A., and Graves, S. S., *J. Am. Chem. Soc.*, 1913, xxxv, 1594

TABLE III.

Guinea Pig Muscle.

3.190	1	26.8	
3.063	1	26.4	
2.587	1	26.1	
2.524	1	26.3	
3.246			13.0
2.591			13.9
4 hrs. later.			
1.887	1	30.2	
2.727	1	30.1	
2.129			16.4
2.223			16.1

TABLE IV.

Cat Muscle.

Weight.	Urease acted.	Mg. per 100 gm. moist muscle.	
		Urea and NH_3 N.	NH_3 N.
gm.	*hrs.*		
2.515	½	24.9	
2.166	½	25.7	
2.560	½	24.8	
3.193	½	25.3	
2.632	2	24.7	
2.001			13.0
4 hrs. later.			
2.990.	½	24.6	
1.973	½	24.0	
2.154	2	25.0	
2.000			15.0

TABLE V.

Guinea Pig Muscle.

2.453	½	26.5	
2.695	½	27.0	
2.717	½	26.5	
2.590			12.8
2.259			13.0
4 hrs. later.			
2.271	½	27.6	
2.176	½	28.0	
2.378	½	27.9	
2.316			13.8
2.310			13.8

TABLE VI.

Cat Muscle.

1.958	½	32.1	
2.266	½	32.7	
1.678	1	32.3	
2.266			12.6
2.217			12.1
4 hrs. later.			
2.060	½	31.5	
1.975	½	31.2	
2.593	1	31.0	
2.302	1	31.4	
2.419			13.0
1.625			14.5

CONCLUSION.

It is noteworthy that the ammonia content of muscle should be so much higher than that of blood, the ammonia content of cat's blood being usually less than 1 mg. per 100 cc. Marshall and Davis[7] give figures for urea in the various tissues of dogs but make no mention of having made analyses for ammonia, or of having taken this into account.

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[7] Marshall and Davis, *J. Biol. Chem.*, 1914, xviii, 53.

To avoid changes that might take place in chopped muscle, analyses were always started as quickly as possible, the urease being added usually within 40 minutes after the death of the animal. In order to observe whether any changes which would affect the figures for urea and ammonia did take place within the first few hours, I have, in a number of experiments, allowed the muscle to stand for a period of about 4 hours at room temperature (24–30°C.) and then carried out other analyses. The results show that there always occurs a slight increase in ammonia but do not allow any conclusion to be drawn with respect to the urea content except that its changes are inconsiderable.

The results published in this paper are believed to show that the determination of urea and ammonia in small samples of muscle can be carried out quickly and accurately by the method described.

THE CHYMASE OF SOLANUM ELAEAGNIFOLIUM.

A PRELIMINARY NOTE.

By A. BODANSKY.

(*From the Department of Physiology and Biochemistry, Medical College, Cornell University, Ithaca.*)

(Received for publication, July 27, 1916.)

In the fall of 1915 the writer's attention was called to the use by the Mexicans of New Mexico and Arizona of the berry of a certain weed as a substitute for rennet in the coagulation of milk. The plant was identified by Professor K. M. Wiegand, of the Department of Botany of the New York State College of Agriculture, as *Solanum elaeagnifolium* Cav.[1] The widespread occurrence of vegetable chymases[2] suggested the presence of a chymase in the berry of this plant, and the writer undertook to test the supposition.[3]

The entire berry of *Solanum elaeagnifolium* was used. It was collected in the late fall and was air-dry when received at the laboratory. After a few preliminary tests of the powdered berry and of its aqueous extract, which gave positive results, a more stable solid preparation was made as follows.

The berries were ground and the coarse powder obtained was extracted by percolation with a 5 per cent sodium chloride solution, containing a few drops of the essential oil of mustard, at a temperature of 5–10°C., after preliminary maceration for 24 hours at the same temperature. The liquid extract was clear, the first portions being brownish green in color, and the subsequent portions yellow. This extract was poured into thirty volumes of acetone, with constant shaking. The precipitate settled rapidly, leaving a clear supernatant liquid. The precipitate was transferred to a Buchner filter, sucked dry, then dried *in vacuo* over sulfuric acid. The powder obtained went easily into solution in water.[4]

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[1] Wooton, E. O., and Standley, F. C., *Contrib. U. S. Nat. Herbarium*, 1915, xix, 573.

[2] Gerber, C., *Compt. rend. Acad.*, 1907–1913, and *Compt. rend. Soc. biol.*, 1907–1913; a summary of the investigations of vegetable chymases is contained in J. Effront's Les Catalyseurs biochimiques dans la vie et dans l'industrie, Paris, 1914, pp. 89 ff.

[3] The writer is indebted to Mr. L. E. Freudenthal, of Solomonville, Ariz., for the information which suggested the present work. The supply of the plant used in the preliminary experiments was obtained through the courtesy of Mr. Ph. Freudenthal.

In order to obtain comparable results in tests of the enzyme preparation, a milk of uniform composition was required. An artificial milk, similar to the one used by Blum and Fuld for testing gastric juice,[5] was prepared by mixing one part of powdered skimmed milk (Merrell-Soule) with nine parts of water, filtering the mixture through a thin layer of cotton, heating it to 80°C.-cooling in running water, and adding calcium chloride. In the earlier tests 0.5 cc. of a 20 per cent solution of calcium chloride was added to 100 cc. of artificial milk. The milk so prepared coagulated after a few minutes' boiling without the addition of enzyme, and was unsatisfactory for tests above 55°C. Satisfactory results were obtained by the use of 0.2 cc. of the 20 per cent calcium chloride solution in 100 cc. of artificial milk.[6]

The properties of the chymase of *Solanum elaeagnifolium* agreed, in general, with the properties of vegetable chymases described by Gerber.[2] It coagulated boiled natural milk without addition of calcium chloride. It proved more resistant to heat than animal rennin and obeyed the law of Segelcke-Storch within the lower range of temperature (up to about 55°C.).

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[4] The method of extraction was essentially the same as that employed by Gerber (*Compt. rend. Soc. biol.*, 1909, lxvi, 890). The use of acetone to precipitate the mixture containing the enzyme is an adaptation of the method of D. D. Van Slyke and G. E. Cullen (*J. Biol. Chem.*, 1914, xix, 211) for the preparation of urease.

[5] Blum, L., and Fuld, E., *Berl. klin. Woch.*, 1905, xliv, 105, quoted by J. Effront (the original publication was not available).

[6] Blum and Fuld recommended the addition of 2 cc. of 20 per cent calcium chloride solution to 98 cc. of artificial milk. J. Wohlgemuth (Grundriss der Fermentmethoden, Berlin, 1913, p. 164), recommends the addition of 0.5 cc. of 20 per cent calcium chloride solution to 100 cc. of artificial milk. Gerber (*Compt. rend. Soc. biol.*, 1909, lxvi, 891) used boiled natural milk sensitized by the addition of 10 mg. molecules (1.11 gm.) of calcium chloride to the liter, performing his tests at 55°C.

In tests conducted between 37 and 55°C. the time of coagulation was inversely proportional to the quantity of the enzyme, other conditions being constant. Increase of temperature had the effect of increasing the rapidity of coagulation, but it also inactivated the enzyme. The optimum temperature was about 84°C., in a dilution of one part of the solid enzyme preparation to 20,000 parts of milk, coagulation taking place in about 1 minute. In a dilution of 1: 100,000 coagulation occurred in 10 minutes, the enzyme being obviously inactivated in the higher dilution by the longer exposure to heat.

Preliminary tests failed to discover any pronounced peptic or tryptic action of the extract. However, further tests will be necessary to justify a definite conclusion.

Work is in progress to determine the presence in *Solanum elaeagnifolium* of other enzymes than chymase, the distribution of the enzymes in the plant, the relative abundance and activity of the enzymes at various stages of growth, and the influence of various factors upon their action. The use of the berry by the Mexicans and the reported local use of *Galium verum* by cheesemakers of the West of England and of *Pinguicula vulgaris* by those of the Italian Alps[7] suggest the possibility of adapting the enzyme preparation of *Solanum elaeagnifolium* to the use by cheesemakers as a substitute for rennet extract. To investigate this possibility is one of the objects of the work outlined above.

The writer wishes to express his obligation to Professor J. B. Sumner, in whose laboratory this work was carried out, for his helpful suggestions and advice.

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[7] Green, J. R., *Ann. Bot.*, 1893, vii, 112.

THE SEPARATE DETERMINATION OF CHOLESTEROL AND CHOLESTEROL ESTERS IN SMALL AMOUNTS OF BLOOD.

By W. R. BLOOR and ARTHUR KNUDSON.

(*From the Laboratories of Biological Chemistry of the Harvard Medical School, Boston.*)

(Received for publication, August 9, 1916.)

In the course of a study of the lipoids of human blood in normal and pathological conditions[1] it was found in almost all cases that cholesterol maintains a strikingly constant relation to the total fatty acids and to lecithin. Even in severe diabetic lipemia[2] where the amount of lipoids was many times the normal the relationship between cholesterol and the total fatty acids remained constant. The inference seemed justified that cholesterol takes an important part in fat metabolism. The most obvious way in which it would participate in fat metabolism is by its fatty acid esters which are always present in the blood and in most tissues, so that a knowledge of the balance between cholesterol and its esters in blood would be of great value in the study of this function. Such knowledge would also be of value in the study of other functions of cholesterol as, for example, the part which it plays as an antihemolytic[3] where the protective power depends on the amount of free cholesterol present. A method was sought which would permit the separate determination of cholesterol and its esters in small amounts of blood. The only successful means at present known for the separation of cholesterol from its esters is the precipitation of the cholesterol by digitonin as worked out by Windaus[4] and the following method is an adaptation of the digitonin precipitation to small amounts of material. It consists in (*a*) the determination of total cholesterol in an aliquot portion of an alcohol-ether extract of blood and (*b*) the determination of cholesterol esters in another aliquot after precipitation of the free cholesterol by digitonin. The difference between the two values represents free cholesterol.

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[1] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

[2] Bloor, *J. Biol. Chem.*, 1916, xxvi, 417.

[3] Ransom, F., *Deutsch. med. Woch.*, 1901, xxvii, 194.

[4] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

The procedure is as follows:

1. Preparation of the Sample.—An alcohol-ether extract of 3 cc. of whole blood, plasma, or serum is made as described in the method for cholesterol already reported.[5]

2. Determination of Total Cholesterol.—This is made with an aliquot portion (generally 10 cc.) of the alcohol-ether extract according to the above method.[5]

3. Precipitation of Free Cholesterol and Determination of the Cholesterol Esters.—20 cc. of the alcohol-ether extract (or sufficient to contain about 0.5 mg. of combined cholesterol) are measured into a small flat-bottomed Erlenmeyer flask (50 cc.) and 1 cc. of 1 per cent alcoholic solution of digitonin is added. The whole solution is then evaporated just to dryness on the water bath or electric stove. The digitonin combines with the free cholesterol forming digitonin cholesteride while the cholesterol present as ester is not affected. The dried residue in the flask is then extracted by boiling out with successive small amounts of petroleum ether (boiling below 60°C.), filtering the extract through a plug of fat-free cotton in the stem of a small funnel.

In order to get a complete extraction with a small amount of solvent 15 cc. of the petroleum ether are first added, the flask is covered with a small watch glass (to prevent too rapid evaporation), and the whole boiled gently until about half the liquid is gone. The succeeding extractions are made in a similar manner with 7 to 8 cc. of petroleum ether. The petroleum ether dissolves the cholesterol esters but does not dissolve the digitonin precipitate. The combined extracts containing the cholesterol esters are then evaporated just to dryness, and the esters taken up with chloroform as in the method for total cholesterol.[5] The chloroform extracts, together slightly less than 5 cc., are collected in a 10 cc. glass-stoppered cylinder (previously calibrated), cooled, and made up to 5 cc. 5 cc. of a standard cholesterol solution in chloroform (containing 0.5 mg.) are measured into a similar 10 cc. cylinder. To each solution are added 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid, the cylinders stoppered, and the solutions mixed by inverting several times. They are then set away in the dark for 15 minutes after which they are transferred to the cups of the colorimeter and the readings made as usual, setting the standard at 15 mm. Artificial light (a 100 watt nitrogen-filled Tungsten light and "daylight" glass) was used throughout these determinations.

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[5] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

DISCUSSION AND RESULTS.

In working out the method for use the following points required to be determined.

a. Whether digitonin as used in the method gives a sufficiently complete precipitation of the cholesterol so that it could be used for the separation of the minute quantities of cholesterol involved in the determination.

b. Whether the solvent used dissolved the cholesterol esters without dissolving any measurable quantity of the precipitated digitonin cholesteride, or dissolving any other substance which would interfere with the determination.

c. Whether cholesterol esters give quantitative values for the cholesterol which they contain. Although it has been claimed[6] that such was the case it seemed desirable to test the point further.

Tests were made as follows:

(1) Portions of 0.5 to 1.5 mg. of cholesterol in alcohol ether were treated with digitonin according to the directions, the residue extracted, and examined for cholesterol by the colorimetric procedure. No color was found in any case so that the treatment was adequate for the complete isolation of free cholesterol.

The digitonin cholesteride, while insoluble in petroleum ether, was found to be measurably soluble in ether and still more in chloroform.

(2) 5 cc. portions of a solution of cholesterol palmitate containing 1.06 mg. of the ester, equivalent to 0.66 mg. of cholesterol, gave (*a*) by the method for determination of total cholesterol 0.681 and 0.687 mg., (*b*) by the method for determination of cholesterol esters 0.675, 0.681, and 0.681 mg.

(3) 5 cc. of the cholesterol palmitate solution to which was added 0.5 mg. of cholesterol gave by the ester method 0.680 and 0.680 mg. cholesterol.

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[6] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

(4) To 3 cc. of extract of blood plasma, found by the ester method to contain 0.441 mg. of cholesterol as ester, were added 3 cc. of the cholesterol palmitate solution containing 0.396 mg. of combined cholesterol. The whole sample then contained theoretically 0.837 mg. of combined cholesterol. Found: 0.815 mg.

(5) To three 5 cc. samples of plasma extract each containing 0.748 mg. of cholesterol as ester were added respectively 0.5, 1.0, and 1.5 mg. of cholesterol, and the esters then determined as usual. The results obtained were 0.742, 0.754, and 0.742 mg., respectively.

The following separations made on human plasma will show the possibilities of the method.

TABLE I.

Cholesterol and Cholesterol Esters in Blood Plasma (Mg. of Cholesterol per 100 Cc. of Plasma).

Sample.	Total cholesterol.	As ester.	Free.	
				Per cent of total.
Diabetes......	538	245	293	54.5
		250	288	53.5
“	312	108	204	65.4
		111	201	64.4
“	508	215	293	57.7
		218	290	57.1
“	250	102	148	59.2
		100	150	59.9
“	556	358	198	35.6
		362	194	34.9
“	268	166	102	38.1
		168	100	37.3
“	182	116	66	36.3
		114	68	37.4
Normal.......	180	117	63	35.0
“	291	164	127	43.6
“	196	117	79	40.3
“	203	123	80	39.3

In a well planned investigation recently published[7] Mueller has questioned the correctness of the values for cholesterol obtained by colorimetric methods. He finds that while both colorimetric and gravimetric (precipitation with digitonin) methods give the same results with ordinary cholesterol the colorimetric methods give higher results with blood. He comes to the conclusion that there are other substances, probably however, closely related to cholesterol, present in blood which give the color reaction but which do not precipitate with digitonin and that therefore the gravimetric method gives more accurate results. He admits, however, that digitonin may precipitate part of these substances from blood and that the digitonin precipitate so obtained is somewhat soluble in the ether used for washing it (as has been found in the work above). Other points brought out in his investigation are a corroboration of the findings[5] that the alcohol-ether treatment as above performed gives a complete extraction of blood and that the results for cholesterol from this extract are higher than those obtained by other colorimetric methods.

The possibility that substances different from ordinary cholesterol but closely related to it may be present in blood has of course been realized, but in view of the lack of definite knowledge regarding their chemical nature, their behavior with digitonin and the Liebermann-Burchard reagent, or even of their presence, an extended discussion of their influence on the cholesterol determination does not seem advisable.

If, as Mueller claims, these substances give the color reaction but do not precipitate with digitonin (or to an insignificant extent) the values for cholesterol esters obtained by the procedure described above would be relatively higher, and those for free cholesterol relatively lower, since the error produced by these substances would fall on the esters with which they would be included if they were not precipitated by the digitonin. As a matter of fact the opposite appears to be the case, since, as may be seen by the values for normal plasma given in the table, and as will be shown more fully in a later publication, the values for free cholesterol are both relatively and absolutely *higher* than those reported in the literature.[8] Either there are no "other substances" in blood plasma or they behave, when treated with digitonin, in the same way as ordinary cholesterol.

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[7] Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.

The colorimetric methods are the only ones which can be used with small amounts of blood and which are therefore suitable for use in the extended study of conditions in living animals, including human beings. The procedure in these methods is relatively simple, the time required short, and the color a satisfactory one to measure. They give, moreover, higher values than the gravimetric method. Until the presence of these "other substances" is definitely shown and their chemical nature determined, the use of the colorimetric methods for the determination of cholesterol and its esters in blood seems fully justified.

[8] Mueller, *J. Biol. Chem.*, 1916, xxv, 561.

THE ISOLATION OF A GROWTH-PRODUCING SUBSTANCE FROM SHEEP PANCREAS.

By WALTER H. EDDY.

(*From the Chemical Laboratory of the New York Hospital.*)

(Received for publication, July 21, 1916.)

The following account covers the preliminary work done to establish the presence of a vitamine in the water-soluble portion of the alcohol extract of sheep pancreas. Experiments are now in progress to establish more fully its nature and properties.

The first attempt to demonstrate the growth-producing substance failed. The method followed was that recommended by Funk, as follows:

1,500 gm. of sheep pancreas were obtained from freshly killed sheep, minced with a meat chopper, and extracted with 4 liters of 95 per cent alcohol (to which was added enough hydrochloric acid to make the alcohol about 4 per cent HCl). The acid alcohol and pancreas were distributed in mason jars and shaken for 3 hours in the shaking machine. At the end of this period the alcohol extract was filtered off and evaporated nearly to dryness *in vacuo* at a temperature of 38°C. The final syrup was then evaporated to dryness before an electric fan at room temperature. (In all succeeding evaporations the fan method was used, the liquid being placed in shallow dishes and fanned with a continuous stream of air at room temperature.) The residue was then melted at 50°C. on a water bath and taken up with a liter of distilled water. This mixture was filtered first on a hot funnel maintained at 38–40°C. The filtered extract was again evaporated to dryness before the fan and the residue again taken up with *cold* distilled water, and filtered at room temperature. This final water solution was next made up to 5 per cent sulfuric acid strength and precipitated with a 50 per cent solution of phosphotungstic acid in 5 per cent sulfuric. In making this precipitate care was taken to avoid excess of phosphotungstic acid. The precipitate was then filtered off and washed thoroughly with 5 per cent sulfuric acid. After washing, it was mixed in a mortar with solid barium hydrate. After thorough mixing water was added and the entire mixture placed in a mason jar and shaken for 3 hours. At the end of this time the mixture was filtered and the filtrate carefully neutralized with sulfuric acid to remove the excess of baryta. After again filtering to remove the barium sulfate the liquid was evaporated before the fan and the residue taken up in absolute alc hol to free it of inorganic salts. The alcohol solution was then precip tated by means of a saturated alcoholic solution of mercuric chlorid This precipitate was filtered off, suspended in water, and subjected to stream of H_2S until all the mercury was converted into the sulfide. T latter was then removed and the mercury-free liquid treated with silv nitrate to precipitate the purines. These purines were then filtered c in turn and the filtrate which contained an excess of silver nitrate w precipitated with silver-baryta. This last was accomplished by fir determining the presence of enough silver nitrate to yield a brown precip tate on the addition of a saturated solution of barium hydrate. Th silver baryta precipitate was washed thoroughly to free it of NO_3 ions an then suspended in water and the silver converted into the sulfide with H and filtered off. There remained a solution which was supposed to co tain the vitamine more or less contaminated by the presence of Ba, SO and Cl ions. These were carefully eliminated by use of sulfuric aci barium chloride, carbon dioxide gas, and neutral moist silver oxide. T final purified solution was then used in the growth experiments.

Using the material, prepared by this method, the attempt wa made to demonstrate its growth-inducing properties by its in troduction into the basal diet of young male white mice. Fo a basal diet in these experiments and in the successful exper ments with rats, Mendel's casein diet was used. Instead, how ever, of using highly purified reagents the diet was made up c the following:

	gm.
Casein (Merck's technical)	180
Starch (Duryea's corn starch)	325
Granulated sugar	170
Agar	50
Lard (Armour's)	250
Salt mixture	25

The salt mixture was composed as follows:	gm.
$Ca_3(PO_4)_2$	10
K_2HPO_4	37
NaCl	20
Na citrate	15
Mg "	8
Fe "	2
Ca lactate	8

The ration was made into a paste by adding the melted lard to th dry ingredients and working in the agar after softening it with distille water.

The animals were given distilled water to drink, each animal was allowed to eat all he wished, and the average daily consumption was noted. The reserve food was kept in the ice chest until needed and made up fresh at least once in 2 weeks. Fig. 1 indicates the typical results obtained from the fifteen mice used in this experiment.

The results of this experiment indicated clearly that the pancreas extract used contained no vitamine, or at least in too small quantity to produce growth results. The extract was given in 5 cc. doses and contained 0.0021 gm. of solid in each 5 cc. Later the extract was concentrated until 5 cc. contained 0.0123 gm. of solid, without favorable results. It contained only a trace of nitrogen.

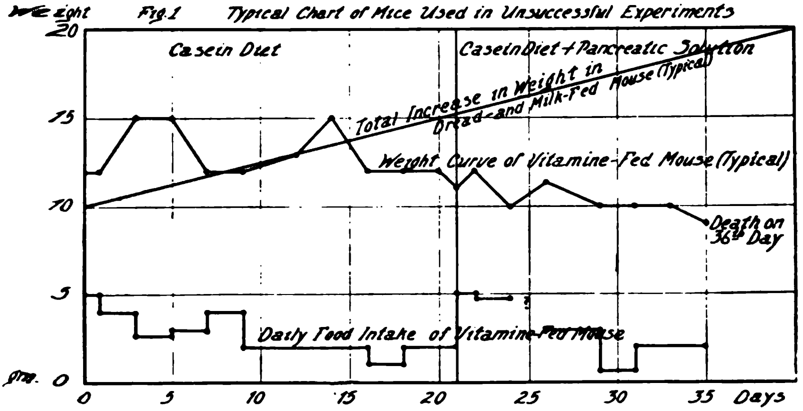

FIG. 1.

Dr. Funk kindly checked the method outlined above and from the results we were led to believe that the failure to obtain the vitamine was due either to the loss of the material in the process of purification or to the small amount of material present. Dr. Funk had meanwhile been able to show that doses of yeast vitamine entirely adequate to cure beri-beri in pigeons were not of sufficient power to produce growth in rats. I therefore decided to repeat the experiments with a larger supply of material and to check the effect of the extract at each step of the purification. These latter experiments have now progressed sufficiently to justify the claim that pancreas does contain the vitamine and in considerable amount. The results to date are given in the following report.

Demonstration of the Presence of Vitamine in Sheep Pancreas.

Materials Used.—3,620 gm. of minced sheep pancreas were extracted with 5,300 cc. of 95 per cent alcohol of 0.8 per cent HCl strength. After thorough extraction the alcohol extract was filtered off and evaporated to dryness before the fan at room temperature. The residue was taken up with distilled water, warmed to 38°C., and the mixture filtered at that temperature on the hot funnel. After again evaporating this filtrate to dryness before the fan the residue was taken up in cold distilled water and this mixture filtered at room temperature. This procedure resulted in a fat- and protein-free liquid which was made up to 2 liters volume with distilled water. This liquid was then divided into four equal portions of 500 cc. each. These four portions were used to prepare the experimental diet solutions as follows:

Part I.—500 cc. of the solution were kept in the ice chest and fed to rats as required.

Part II.—500 cc. of the solution were treated with 25 gm. of Lloyd's reagent.[1] The reagent was shaken with the pancreatic extract and the powder then filtered off and later mixed with the food of the rats. The filtrate was also retained and used in the diet of other rats as a control.

Part III.—In accordance with the method of Jacobs,[2] 500 cc. of the solution were made acid with hydrochloric acid to 5 per cent strength and then precipitated with a solution of 25 per cent phosphotungstic acid in 5 per cent HCl. After washing this precipitate with acid (5 per cent HCl) it was removed to a separatory funnel and into this funnel was put one volume each of amyl alcohol, ether, and water, made acid with HCl. Shaking this mixture caused the phosphotungstic acid portion of the precipitate to pass into solution in the amyl alcohol and the rest of the precipitate passed into solution in the water. This water solution was then filtered off, carefully neutralized with NaOH, and used to feed certain rats.

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[1] Seidell, A. (*U. S. Pub. Health Report,* No. 325, 1916), claims that Lloyd's reagent—colloidal hydrous aluminium silicate—exerts a selective adsorption for vitamine. His experiments were conducted with yeast vitamine.

[2] Jacobs, W. A., *J. Biol. Chem.,* 1912; xii, 429. See also Van Slyke, D. D., *ibid.,* 1915, xxii, 283.

Part IV.—500 cc. were held in reserve.

In the feeding experiments I had then for use the following solutions: A. A filtered water-soluble portion of the alcohol extract of pancreas. B. Lloyd's reagent after shaking with the water-soluble portion of the alcohol extract of pancreas and supposedly containing the adsorbed vitamine. C. Filtrate from the water-soluble portion of the alcohol extract after treatment with Lloyd's reagent. D. The phosphotungstic precipitate of the water-soluble portion of the alcohol extract of pancreas held in neutral water solution after freeing of phosphotungstic acid with amyl alcohol according to Jacobs' method. In the following descriptions these solutions will be designated by the letters used. All the liquids (A, C, and D) and the solid, B, were kept in the ice chest throughout the entire period of the experiment. This was done as a precaution and not because of any definite proof that the substance deteriorates at room temperature.

Demonstration Methods.—For these experiments young male rats (white) were used, ranging from 40 to 90 gm. in weight at the beginning of the experiment. They were kept in separate cages and the casein diet was used as the basal food. In order to make sure that the casein was vitamine-free, all that was used in the food of the rats was first boiled for several hours in 95 per cent alcohol. The food was placed in feeding boxes so arranged as to prevent contamination by feces. Distilled water was given for drinking purposes. Twelve rats were used in the feeding experiments. Four were maintained on a mixed diet of bread, milk, carrots, cabbage, etc., to determine normal growth. Three of these are plotted in Fig. 2 (Rats 6, 7, and 12). One of the four developed scurvy and his curve is not included.

Rats 1 and 2 were fed the casein diet for a period of 16 days. In this time they steadily declined in weight, as shown by the heavy line. (The dotted line shows the average food intake per day.) On the 16th day the casein diet was mixed with 16 cc. of the water-soluble pancreatic extract A. At each succeeding weighing period new food was given mixed with 10 cc. of the extract. The upward rise in the weight curve was i diate and was steadily maintained throughout the entire e mental period of 23 days following the first dose. These r

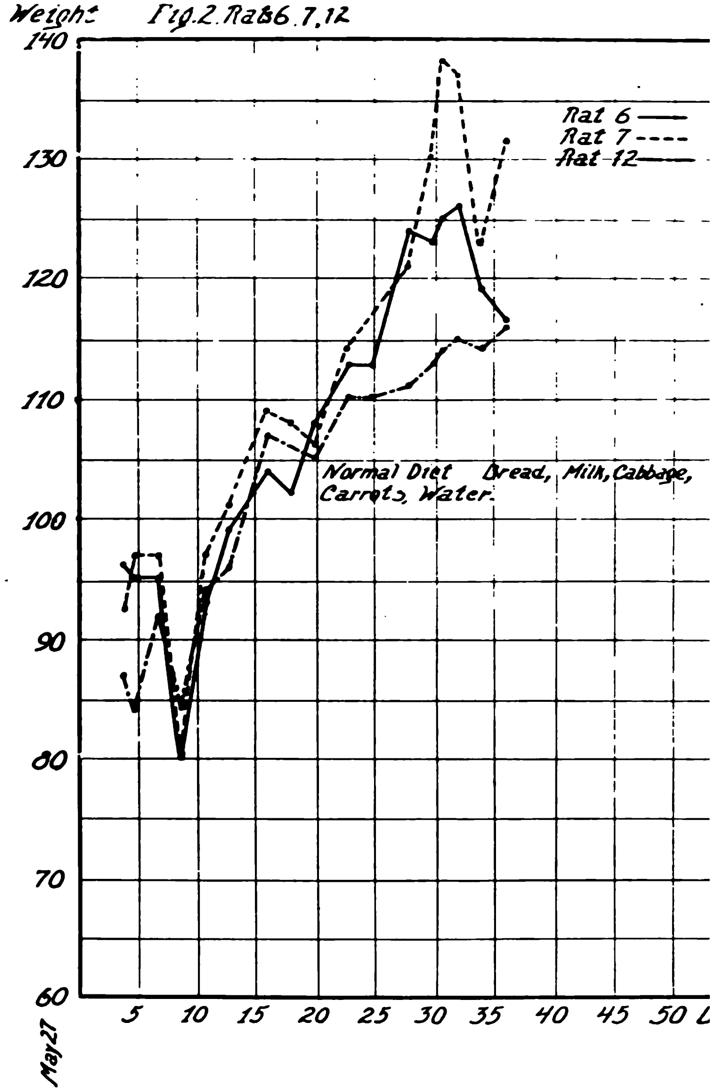

Fig. 2.

indicated clearly the power of the extract to induce growth and also to develop increase in daily food consumption.

As in the case of Rats 1 and 2, the diet of Rat 3 was of the casein

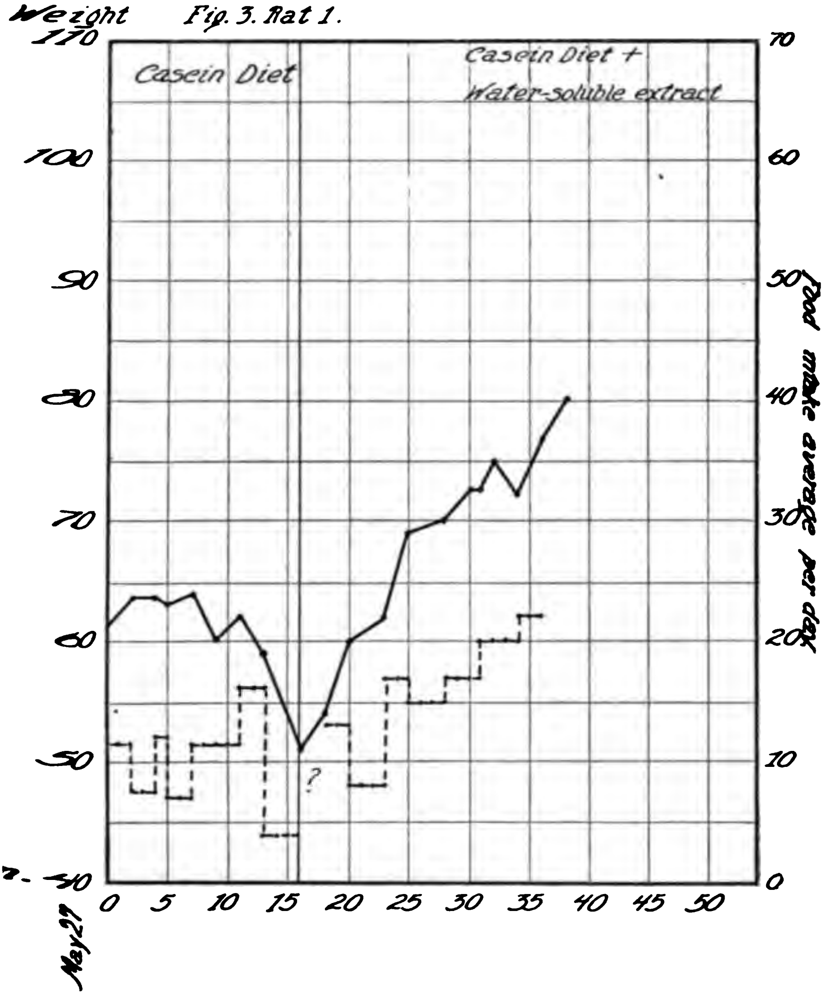

FIG. 3.

mixture for 16 days with bare maintenance of weight for that period. On the 16th day the water-soluble extract A was added and the corresponding rise in weight occurred. On the 25th day the rat was put back on the casein diet and in the next days declined 6 gm. in weight, showing that the presence of t extract was necessary to maintain growth. On the 32nd d

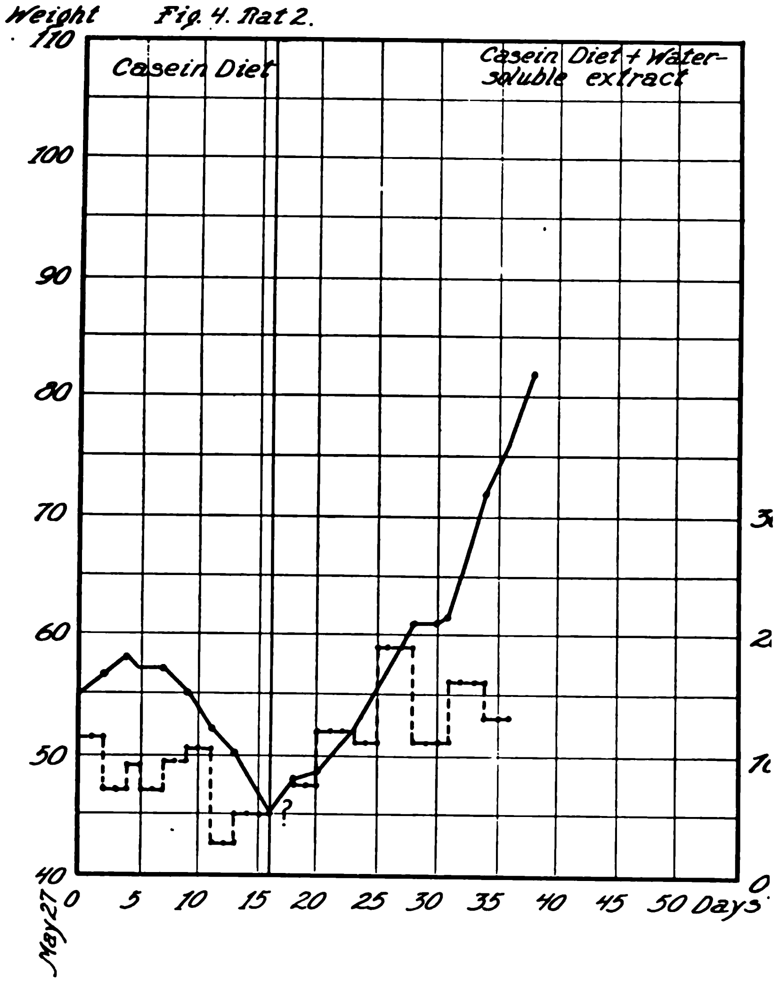

FIG. 4.

this rat was given a dosage of 10 cc. of the phosphotungs precipitate D. The animal immediately began to gain weig and continued to do so to the end of the experiment on the 3 day. This experiment demonstrated that the growth-producing power of the water extract was not lost in the first separation step.

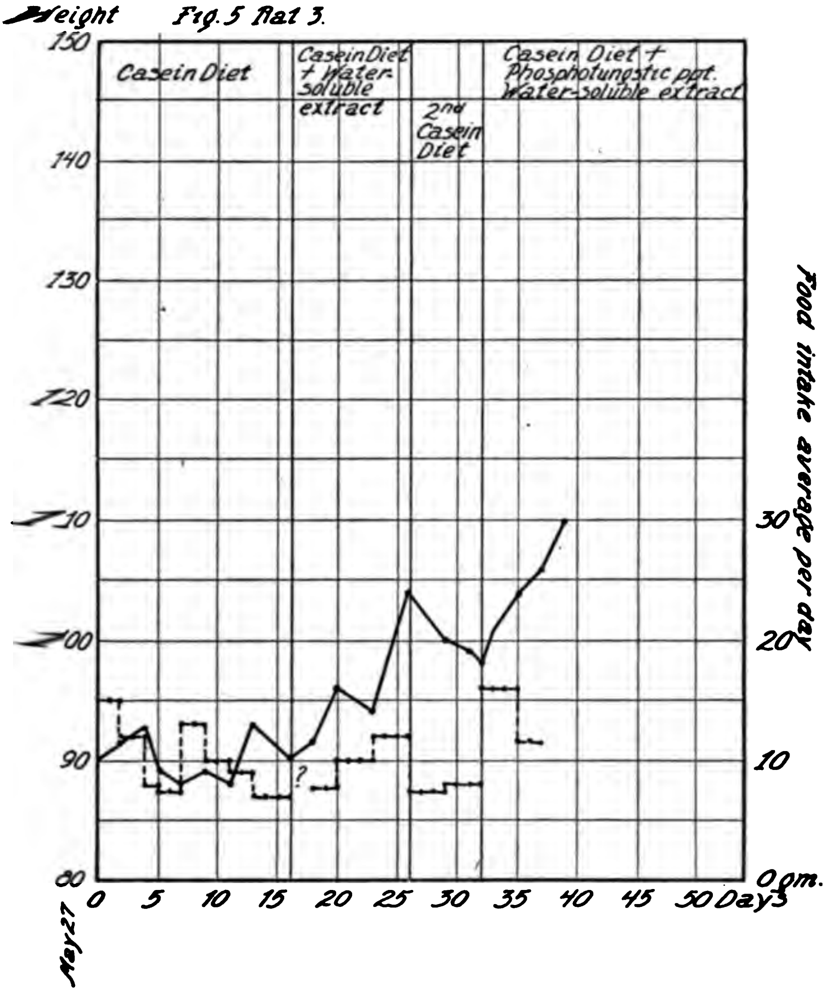

Fig. 5.

Rats 4 and 5 were given 18 days of casein diet with maintenance or decline. On the 18th day 12 gm. of Lloyd's reagent, B, were added to the diet. At each succeeding period 5 to 6 gm.

were added. In the 10 days of this treatment the rats showe an increase in weight, demonstrating the power of this sul stance to adsorb the vitamine and confirming Seidell's[1] concl sions. (Further confirmation of this point was given by Rat 9

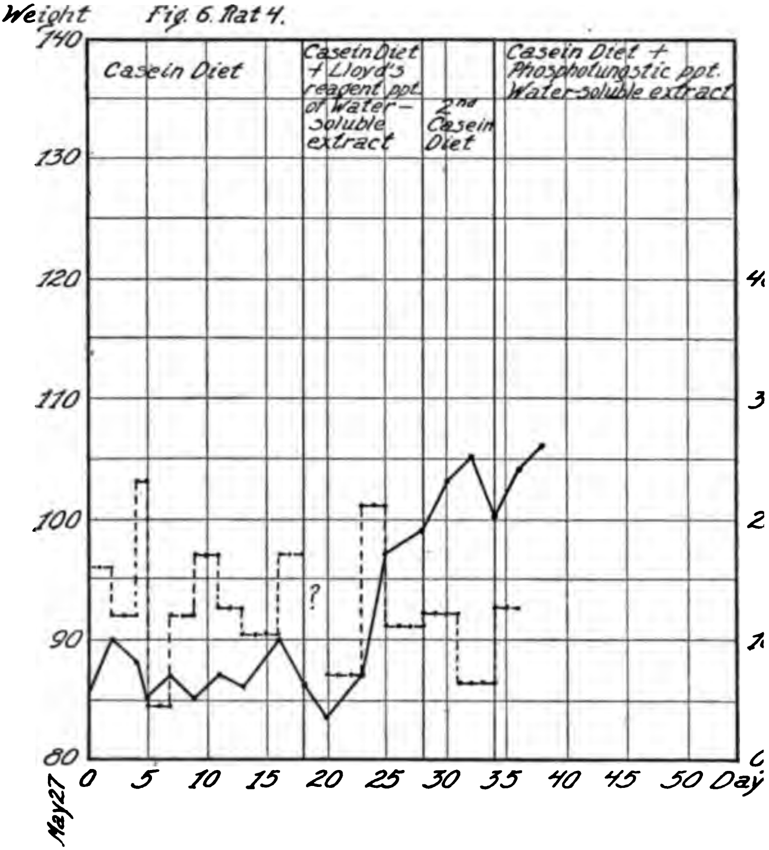

FIG. 6.

On the 28th day these rats were returned to the casein diet a it was interesting to note that Lloyd's reagent was found persist in its stimulating effects for some 3 or 4 days after i feeding had stopped. At the end of 6 days the rats had beg to decline. Both of these rats were made to resume their u ward rise in weight by the addition of 10 cc. doses of the phosphotungstic precipitate D to the diets on the 32nd and 34th days respectively, further confirming the results with Rat 3.

Rat 9 was fed casein diet for 23 days with a marked decline

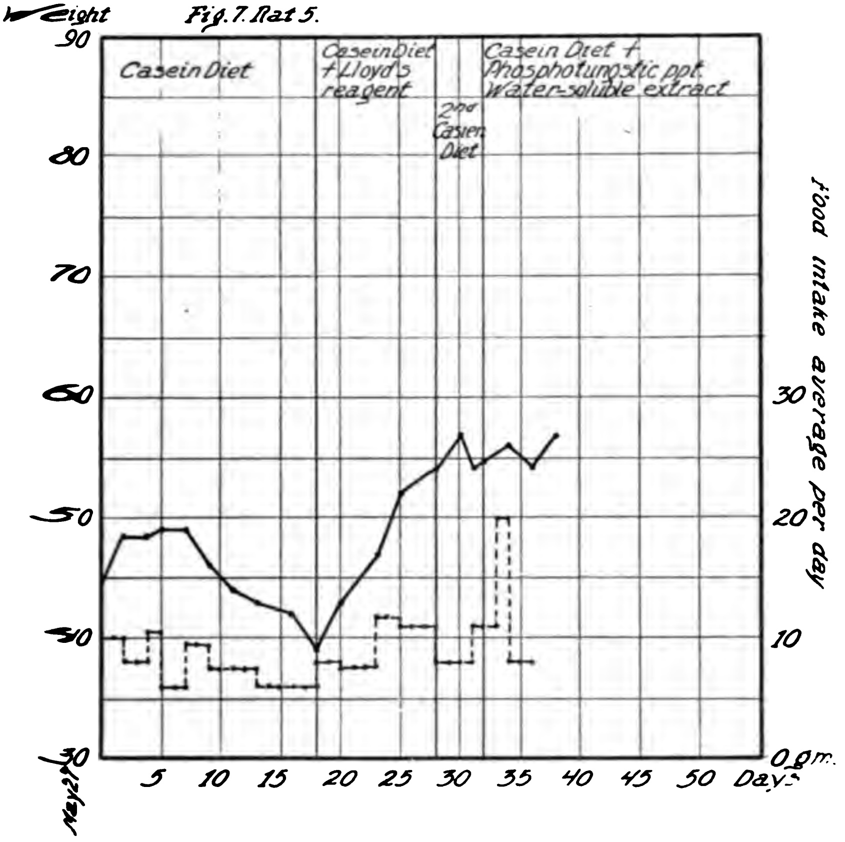

FIG. 7.

in weight. On the 23rd day a dosage of the filtrate C from Lloyd's reagent was begun (10 cc. amounts at each weighing period). This failed to do more than produce a lessening in the decline of the curve, indicating that the Lloyd reagent removes at least the greater part of the vitamine. At the end of 8 days of this diet the rat was given 1 day's ration of casein food alone and then a dosage of the phosphotungstic precipitate was begun (D). Recovery and increase were immediate.

Rat 10 was fed casein diet for 32 days with a decrease in weigh of some 30 gm. In spite of this long period of decline the feeding of the phosphotungstic precipitate D on the 32nd day broug about immediate rise in weight which was maintained.

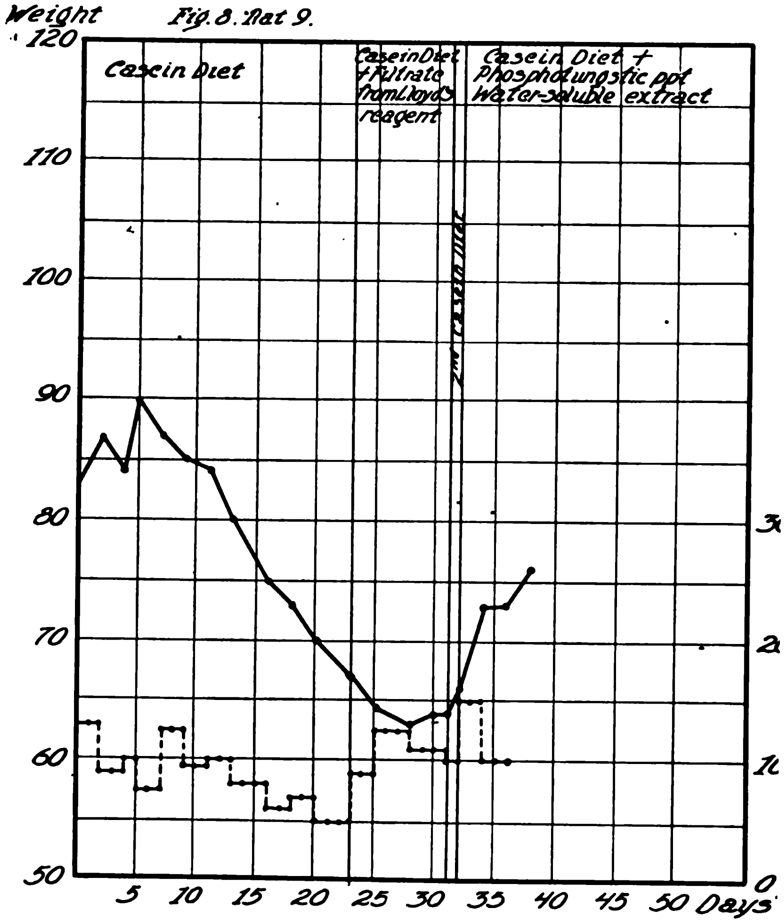

FIG. 8.

At the end of the charted period the rats were all in good co dition except the one control rat mentioned that showed incipie scurvy. None of the extract-fed rats showed any signs of scurvy or of other dietary diseases.

GENERAL CONCLUSIONS.

The results of the rat experiments seemed to prove conclusively that the water-soluble portion of the alcoholic extract of pancreas contains a substance that is capable of inducing marked increase in growth. This substance is removed from the extract without loss of power by treatment with Lloyd's reagent. It is also precipitated in the phosphotungstic precipitate of the extract. It is not a protein or fatty substance. It occurred to me that the results might indicate not a vitamine but a happy

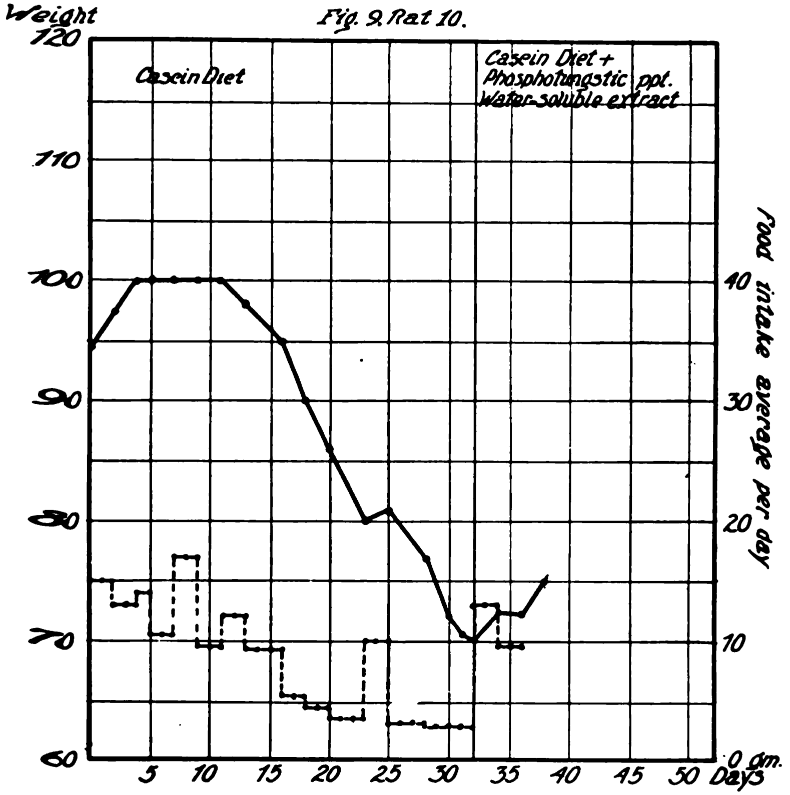

FIG. 9.

combination of amino-acids. To determine this point I secured amino nitrogen determinations on solutions A, C, and D. These solutions were analyzed by Dr. J. C. Bock of Cornell Medical College, to whom they were given as lettered samples. The results of the analyses were as follows:

Solution A contained 4.839 mg. of amino N per cc.
“ C “ 4.854 “ “ “ “ “ “
“ D “ 1.370 “ “ “ “ “ “

Solution C was the solution from which the vitamine was removed with Lloyd's reagent and had practically no growth-stimulating power. The result of the above analysis indicates that the Lloyd powder removed none of the amino-acids, yet did remove the growth stimulus. The fact that the phosphotungstic solution D contained much less amino-acid and yet was powerful in a growth stimulation also tends to make the conclusion stronger that we were dealing with something other than amino-acid stimulus.

In conclusion I wish to acknowledge my indebtedness to Dr. Nellis B. Foster, to Dr. Wm. J. Elser, and to Dr. Stillman for the facilities given me at the New York Hospital, to Dr. Casimir Funk for his suggestion and criticism throughout the research, and to Dr. J. C. Bock for his assistance in the matter of the amino-acid determinations.

STUDIES IN CREATINE METABOLISM.*

I. POSSIBLE INTERRELATIONS BETWEEN ACIDOSIS AND CREATINE ELIMINATION.

By FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, July 31, 1916.)

Current views[1] associate the elimination of creatine with some perversion of carbohydrate metabolism. The probability of a close relationship of this sort is indicated by the well known fact that a deficiency of carbohydrate in the body, as in starvation, leads to creatine elimination which may be checked promptly by ingestion of carbohydrate. Depletion of the body of carbohydrate may also be accomplished through the action of various toxic substances like phlorhizin, phosphorus, hydrazine, etc., or through disease as in diabetes. In each of the enumerated instances of carbohydrate deficiency, creatine appears in the urine. There are, however, experimental facts which the familiar hypothesis fails to explain. McCollum and Steenbock[2] found that in the pig a diet of corn products, among others, led to the appearance of relatively large quantities of creatine in the urine. The authors point out that the "character of the proteins in the diet appears to determine whether a large or a small creatine production takes place in the tissues. These observations leave little doubt that creatine may come from exogenous as well as endogenous protein metabolism." Somewhat similar experiments of Folin[3] with oat feeding yielded comparable results. The dietaries employed, especially those of McCollum and Steenbock, cannot be regarded as lacking in carbohydrate, for in the creatine-producing diet was included a significant quantity of added starch.

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* The results of this investigation were reported to the Society for Experimental Biology and Medicine, March 15, 1916. See *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 113.

[1] Recent literature concerning creatine may be found in the papers of Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213; Riesser, O., *Z. physiol. Chem.*, 1913, lxxxvi, 415; Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xiv, 9; 1913, xv, 283, 285; 1913–14, xvi, 169; 1915, xxi, 377, 383, 389. Benedict, S. R., and Osterberg, E., *ibid.*, 1914, xviii, 195.

[2] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.

Deficiency of carbohydrate usually means an accompanying acidosis, not necessarily caused by ketogenic substances, which presumably involves the tissues associated with creatine-creatinine metabolism. At any rate nearly every instance in which there is creatine in the urine is accompanied by an acidosis—generally a ketonuria also. The term acidosis in the sense used here means a disturbance in the acid-base equilibrium in the body, whereby the excessive production of acid or introduction of substances forming an acid ash causes the withdrawal of base from the body. In man it is probable that in general the body neutralizes such acid with ammonia, the neutralization of acid with other bases never having been demonstrated. Carnivora undoubtedly neutralize acids in a similar manner. With the rabbit, however, the neutralization of acid may not necessarily be performed by ammonia, for in experimental acid poisoning in these animals there is not a close relationship between the output of ammonia and the introduction of acid, but the other bases as sodium, potassium, calcium, and magnesium, are much increased in the urine. With the dog, on the other hand, such a relationship obtains and the other alkali bases are relatively little increased. The older explanation of these facts was to the effect that the herbivorous animal ingested too little protein material to furnish sufficient ammonia for neutralization purposes. Another possible viewpoint is that owing to the general character of the diet consumed by the herbivora a great excess of base is introduced into the body, as indicated by the general alkalinity of the urine of herbivora, which may be readily available for neutralization purposes under ordinary conditions. The store of such available base must be somewhat limited, however, since the great susceptibility of the rabbit to acid is well known. In view of this fact it would seem that in questions involving the influence of acid upon varying phases of metabolic activity the rabbit would serve as an ideal experimental animal, much more so than the dog, which is very resistant to acid introduction.

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[3] Reported at the meeting of the American Society of Biological Chemists, December, 1915.

The fact that in nearly every instance in which creatine appears in the urine there is an accompanying acidosis suggests the hypothesis that a condition of acidosis in the body is responsible for the appearance of creatine in the urine. To test it the following questions demand an answer. 1. If acidosis is induced, will creatine appear in the urine, even in the presence of an adequate carbohydrate supply? 2. Will the elimination of creatine disappear if the acidosis is abolished, quite independently of the factor of carbohydrate supply?

In the present paper a condition of acidosis was considered indicated when the urine of the rabbit became strongly acid, as shown by determination of the hydrogen ion concentration, employing sodium alizarine sulfonate as the indicator.[4] In the sense of the term acidosis as used here, that is, a condition of alkali depletion, the assumption given above is undoubtedly correct since ordinarily the rabbit secretes urine which is strongly alkaline.

The general plan of the investigation was as follows: Rabbits were fed upon diets containing adequate carbohydrate supply but of such a nature that one diet would yield a pronounced acid ash, another a distinctly basic ash, and finally the third type of diet consisted of a mixture of the other two. The acid-producing foods were whole oats and cracked corn, the mixture being designated the "grain diet." Fresh carrots constituted the base-producing food, designated "carrot diet." A dietary containing oats, corn, and carrots is called the "mixed diet."

The results obtained are to be found in Tables I to VI. Upon a diet of oats and corn containing an adequate supply of carbohydrate, creatine[5] promptly appears in the urine. A markedly increased hydrogen ion concentration of the urine is always associated with this phenomenon. The presence of creatine in the urine is just as easily manifested upon a grain diet whether the latter is given to animals fresh from stock or after a relatively prolonged period in which the base-producing foods have predominated. Upon the grain diet the urine of the two rabbits tested, Tables VI and VII, acquired distinct tests for protein. The condition of albuminuria persisted throughout the period of observation. It is of considerable significance in the etiology of nephritis that *protein may appear in the urine as a result of a change in the character of the diet.*

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[4] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393. The presence of phosphates in the alkaline urines interfered with accurate determinations of the hydrogen ion concentration; hence the figures given for the alkaline urines are only approximations.

[5] 24 hour specimens of urine were obtained by compression of the bladder through the abdominal wall. Creatinine was estimated according to the method of Folin and creatine by the procedure of Benedict and Myers.

Upon a carrot diet urinary creatine rapidly disappears as the urine becomes alkaline; or if creatine was originally absent from the urine it remains so.

Under the conditions outlined above the facts with regard to creatine elimination may be interpreted in two ways, first, the appearance of creatine in the urine on the grain diet and its disappearance on the carrot diet may be ascribed to the character of the proteins constituting the dietaries, or second, the phenomenon under discussion may be explained on the basis of the acid-base-producing potentialities of the dietaries. To determine the responsible factor animals were fed upon the "mixed diet" constituted by a mixture of the two types of dietaries. From the tables it is obvious that the protein *per se* in the diet is without special significance for upon the mixed diet creatine fails to appear in the urine and the reaction of the latter remains alkaline. The acid-producing power of the grain diet must therefore be considered as the potent factor in the production of creatinuria under the experimental conditions. To demonstrate still further the validity of this inference rabbits, maintained upon a "mixed diet," hence consequently without creatinuria and with alkaline urines, were given relatively small doses of hydrochloric acid by means of the stomach sound twice daily. This addition of hydrochloric acid to the "mixed diet" without change of proteins causes the appearance in the urine of significant quantities of creatine. Simultaneously the hydrogen ion concentration of the urine is markedly increased.

The facts enumerated above also confirm the findings of McCollum and Steenbock that creatine may appear in the urine even when adequate carbohydrate is supplied; and they lead to the conclusion that creatinuria is not necessarily related to carbohydrate deficiency in the body. It would appear to be associated with a condition of acidosis which may and usually does accompany those states of metabolism in which carbohydrate deficiency is especially noticeable.

In general, the figures for creatinine are fairly constant for each individual animal, but an inspection of the tables will show that there is on the average a decided tendency for creatinine to be increased on the carrot diet and to be decreased when hydrochloric acid is added to the mixed diet.

The tables follow.

TABLE I.

The Relation of the Character of the Food Intake to P_H *of the Urine and to the Elimination of Creatine.*

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Jan. 24	90	1.025	135	0	8.70	White male rabbit of 2,400 gm.
" 25	65	1.023	92	18	6.70	Grain diet { 50 gm. oats. 50 " cracked corn.
" 26	100	1.030	72	45	6.30	
" 27	60	1.035	90	34	5.30	
" 28	30	1.040	81	27	4.90	
" 29	50	1.030	81	31	4.70	Feces soft.
" 30	60	1.025	81	39	4.70	" "
" 31	60	1.024	77	39	4.70	" "
Average			88			
Feb. 1	60	1.030	81	13	5.30	Carrot diet: 300 or 500 gm. carrots. For first 2 days 300 gm. carrots were given. From this period on the animal received 500 gm. carrots.
" 2	300	1.010	90	5	7.38	
" 3	300	1.010	81	0	7.48	
" 4	300	1.010	93	0	7.48	
" 5	425	1.012	91	0	7.48	
" 6	275	1.025	92	0	8.0	
" 7	300	1.016	100	0	9.27	
" 8	350	1.015	90	0	8.70	
Average			90			
Feb. 9	250	1.020	73	0	8.70	Mixed diet { 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 10	120	1.015	88	0	8.70	
" 11	120	1.015	90	0	8.70	
Average			83			
Feb. 12	170	1.012	57	0	8.0	12 m. 50 cc. 0.25 N HCl by stomach tube.
" 13	130	1.010	66	10	4.90	11 a.m. 50 cc. 0.25 N HCl by stomach tube. 6 p.m. 50 cc. 0.25 N HCl by stomach tube.
" 14	150	1.020	55	45	6.00	11 a.m. 50 cc. 0.25 N HCl by stomach tube. 6 p.m. 50 cc. 0.25 N HCl by stomach tube.
Average			59			

TABLE II.

The Relation of the Character of the Food Intake to P_H *of the Urine and to the Elimination of Creatine.*

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Jan. 24	50	1.038	95	0	8.70	Male rabbit of 2,200 gm.
" 25	60	1.035	95	11	6.70	
" 26	50	1.032	83	51	6.30	Grain diet: 50 gm. oats. 50 " cracked corn.
" 27	50	1.030	101	39	5.30	
" 28	40	1.035	81	36	5.70	
" 29	50	1.035	108	45	4.90	
" 30	50	1.035	81	45	4.70	
" 31	55	1.032	81	39	4.90	
Feb. 1	50	1.035	81	57	4.70	Urine showed distinct test for protein.
" 2	50	1.035	90	41	4.70	Previous to experiment urine was protein-free.
Average			89			
Feb. 3	110	1.020	97	31	4.90	Carrot diet for first 2 days 300 gm.
" 4	400	1.015	116	18	7.48	Carrot diet for remainder of period, 500 gm.
" 5	425	1.012	112	0	7.48	
" 6	275	1.025	104	0	8.0	
" 7	400	1.012	98	0	8.70	
" 8	350	1.015	113	0	8.70	Urine still shows traces of protein.
Average			106			
Feb. 9	170	1.020	94	0	8.70	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 10	100	1.025	70	0	8.70	
" 11	120	1.020	88	0	8.70	
Average			84			
Feb. 12	150	1.012	71	14	6.30	12 m. 50 cc. 0.25 N HCl by stomach tube.
" 13	110	1.020	68	68	4.90	11 a.m. 50 cc. 0.25 N HCl by stomach tube. 6 p.m. 50 cc. 0.25 N HCl by stomach tube.
" 14						Animal killed because of injury.
Average			69			

TABLE III.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Jan. 24	60	1.035	95	0	8.70	Male rabbit of 2,200 gm.
“ 25	50	1.030	90	0	6.90	
“ 26	90	1.030	109	53	6.00	
“ 27	60	1.030	85	52	4.90	Grain diet: 50 gm. oats. 50 “ cracked corn.
“ 28	60	1.025	112	27	4.90	
“ 29	70	1.025	81	63	4.70	
“ 30	60	1.030	81	49	4.70	
“ 31	70	1.025	81	57	4.70	
Feb. 1	70	1.025	81	63	4.70	
“ 2	60	1.030	90	52	4.70	Urine previously protein-free contained distinct traces of protein.
Average			**90**			
Feb. 3	150	1.015	95	57	5.70	Carrot diet for first 2 days, 300 gm.
“ 4	470	1.015	139	10	7.38	Carrot diet for remainder of period, 500 gm.
“ 5	400	1.012	112	0	7.48	
“ 6	375	1.015	131	0	8.0	
“ 7	400	1.012	114	0	8.70	
“ 8	375	1.015	120	0	8.70	Urine still contains protein.
Average			**118**			
Feb. 9	190	1.018	118	0	8.70	Mixed diet: 25 gm. oats. 25 “ cracked corn. 250 gm. carrots.
“ 10	120	1.030	97	0	8.70	
“ 11	170	1.015	100	0	8.70	
“ 12	250	1.010	115	0	8.0	
Average			**107**			
Feb. 13	120	1.015	97	49	4.90	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
“ 14	170	1.015	93	70	4.90	
“ 15	160	1.015	92	43	4.70	
Average			**94**			
Feb. 16	425	1.015	100	13	9.27	Carrot diet: 500 gm. carrots.
“ 17	425	1.012	172	0	9.27	
“ 18	350	1.015	125	0	9.27	
“ 19	400	1.015	134	0	9.27	
“ 20	250	1.020	113	0	9.27	
“ 21	120	1.035	114	0	9.27	
Average			**126**			

TABLE III.—*Concluded.*

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P$_H$.	
1916	*cc.*		*mg.*	*mg.*		
Feb. 22	275	1.015	113	23	7.38	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	120	1.025	100	72	5.70	
" 24	175	1.015	100	34	5.30	
Average			**104**			
Feb. 25	350	1.010	133	28	9.27	Carrot diet: 500 gm. carrots.
" 26	465	1.013	128	0	9.27	
" 27	325	1.017	114	0	9.27	
Average			**125**			
Feb. 28	80	1.035	136	0	6.90	Grain diet: { 50 gm. oats. 50 " cracked corn.
" 29	70	1.035	103	17	4.70	
Mar. 1	96	1.020	110	34	4.70	
" 2	90	1.015	121	34	4.70	
" 3	93	1.020	126	14	4.70	
Average			**119**			
Mar. 4	265	1.010	130	0	9.27	Carrot diet: 500 gm. carrots. Body weight at end of experiment, 2,280 gm.
" 5	430	1.015	138	0	9.27	
Average			**134**			

TABLE IV.

The Relation of the Character of the Food Intake to P_H *of the Urine and to the Elimination of Creatine.*

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	cc.		mg.	mg.		
Feb. 16	325	1.017	132	36	9.27	Male rabbit of 2,700 gm. Previous diet unknown.
" 17	350	1.015	123	14	9.27	
" 18	325	1.015	104	0	9.27	Carrot diet: 500 gm. carrots.
Average			119			
Feb. 19	400	1.015	134	0	9.27	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 20	140	1.025	113	0	9.27	
" 21	90	1.038	121	0	9.27	
Average			123			
Feb. 22	235	1.015	126	0	7.38	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	275	1.012	99	98	5.30	
" 24	235	1.015	118	24	5.30	
Average			114			
Feb. 25	275	1.015	115	16	9.27	Carrot diet: 500 gm. carrots.
" 26	310	1.015	119	7	9.27	
" 27	360	1.012	115	0	9.27	
Average			116			
Feb. 28	65	1.035	114	0	6.70	Grain diet: 50 gm. oats. 50 " cracked corn.
" 29	80	1.040	118	18	4.70	
Mar. 1	81	1.040	100	53	4.70	
" 2	73	1.025	99	54	4.70	
" 3	84	1.019	113	33	4.70	
Average			108			
Mar. 4	150	1.015	111	0	6.0	Carrot diet: 500 gm. carrots.
" 5	410	1.015	129	0	9.27	
Average			120			

TABLE V.

The Relation of the Character of the Food Intake to P_H of the Urine and to the Elimination of Creatine.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Feb. 16	400	1.015	94	0	9.27	Male rabbit of 2,400 gm. Previous diet unknown.
" 17	450	1.015	144	0	9.27	
" 18	350	1.018	88	0	9.27	Carrot diet: 500 gm. carrots.
Average			**108**			
Feb. 19	400	1.015	110	0	9.27	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 20	100	1.026	84	0	9.27	
" 21	150	1.025	96	0	9.27	
Average			**97**			
Feb. 22	330	1.013	102	33	7.38	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	255	1.013	98	50	5.70	
" 24	250	1.012	100	30	5.30	
Average			**100**			
Feb. 25	350	1.012	112	14	9.27	Carrot diet: 500 gm. carrots.
" 26	450	1.015	122	0	9.27	
" 27	390	1.013	86	0	9.27	
Average			**107**			
Feb. 28	90	1.025	121	0	6.90	Grain diet: 50 gm. oats. 50 " cracked corn.
" 29	60	1.035	81	7	4.70	
Mar. 1	60	1.025	88	37	4.70	
" 2	90	1.023	91	64	4.70	
" 3	68	1.025	92	35	4.70	
Average			**95**			
Mar. 4	340	1.010	107	14	9.27	Carrot diet: 500 gm. carrots.
" 5	400	1.016	108	0	9.27	
Average			**107**			

TABLE VI.

The Relation of the Character of the Food Intake to P_H *of the Urine and to the Elimination of Creatine.*

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Creatinine.	Creatine.	P_H.	
1916	*cc.*		*mg.*	*mg.*		
Feb. 16	300	1.016	100	0	9.27	Male rabbit of 2,000 gm. Previous diet unknown.
" 17	400	1.015	108	0	9.27	
" 18	325	1.015	89	0	9.27	Carrot diet: 500 gm. carrots.
Average			**99**			
Feb. 19	325	1.020	80	0	9.27	Mixed diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 20	130	1.028	98	0	9.27	
" 21	130	1.020	83	0	9.27	
Average			**87**			
Feb. 22	130	1.020	85	0	7.48	Each day the animal received 100 cc. 0.25 N HCl divided into two equal doses, one being given at 11 a.m., the other at 6 p.m.
" 23	275	1.010	77	15	5.70	
" 24	180	1.017	76	31	5.30	
Average			**80**			
Feb. 25	275	1.015	91	6	9.27	Carrot diet: 500 gm. carrots.
" 26	425	1.015	92	0	9.27	
" 27	320	1.012	96	0	9.27	
Average			**93**			
Feb. 28	80	1.025	91	0	7.38	Grain diet: 25 gm. oats. 25 " cracked corn. 250 gm. carrots.
" 29	65	1.030	81	34	5.30	
Mar. 1	45	1.040	90	46	5.30	
" 2	70	1.026	90	42	5.30	
" 3	60	1.020	65	50	5.30	
Average			**83**			
Mar. 4	180	1.015	70	23	9.27	Carrot diet: 500 gm. carrots.
" 5	340	1.018	82	0	9.27	
Average			**76**			

SUMMARY.

Upon a diet of oats and corn, containing an adequate supply of carbohydrate, creatine promptly appears in the urine of the rabbit. A marked condition of acidosis, as measured by the hydrogen ion concentration of the urine is always associated with this phenomenon. Oats and corn are pronounced acid-producing foods. On the other hand, if a base-producing food, such as carrots, is fed to rabbits with creatinuria, this symptom rapidly disappears as the urine becomes alkaline.

The protein *per se* is without special significance in the phenomenon under discussion; for upon a diet consisting of oats, corn, and carrots creatine fails to appear in the urine, and the reaction of the latter remains alkaline. Equally significant is the further fact that the ingestion of hydrochloric acid with the mixed diet causes the appearance in the urine of significant quantities of creatine. Simultaneously the hydrogen ion concentration of the urine is markedly increased.

The conclusion seems justified that there is an interrelationship between acidosis and creatine elimination. Creatine in the urine may prove to be an index of a condition of acidosis in the organism.

STUDIES IN CREATINE METABOLISM.

II. THE INFLUENCE OF ALKALI UPON CREATINE ELIMINATION DURING INANITION.

By FRANK P. UNDERHILL.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.*)

(Received for publication, July 31, 1916.)

It is well recognized that during inanition creatine appears in the urine of the rabbit in significant quantities and it is likewise well known that in a starving condition the urine becomes markedly acid in reaction. This change in reaction of the urine has been explained by the assumption that the starving herbivorous animal feeds upon its own tissues in an effort to derive sufficient fuel for its needs and under these circumstances is to all intents and purposes with respect to the character of its metabolism a carnivorous animal. During the process of disintegration of tissue thus devoted to calorific needs acids are formed which must be neutralized by fixed alkalies of the organism and this continual demand for alkali results in base depletion eventually—a concomitant of which is the elimination of creatine in the urine.

According to these ideas, then, in starvation in the rabbit there should be a greatly increased hydrogen ion concentration in the urine. That such a condition obtains may be seen from the data in Table I. Here it is shown that creatinuria and a markedly high hydrogen ion concentration occur simultaneously. These results are in agreement with the hypothesis advanced in the preceding paper; namely, that creatine in the urine is associated with a condition of acidosis and may be independent of the carbohydrate supply. On the other hand, during the creatinuria of inanition in the rabbit[1] and in pernicious vomiting of pregnancy[2] carbohydrate introduction into the body will either greatly diminish or totally inhibit the excretion of creatine. This may be explained by assuming that there is diminished tissue breakdown after carbohydrate introduction, hence decreased acid formation, so that base depletion is inhibited more or less completely.

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[1] Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213.

[2] Underhill, F. P., and Rand, R. F., *Arch. Int. Med.*, 1910, v, 61.

If these ideas are correct, and in particular the view that base depletion is responsible for creatine elimination, it would appear reasonable to assume that during creatinuria, as in inanition, administration of base should either markedly decrease or entirely prevent the appearance of creatine in the urine. The results of four such experiments may be found in Tables II, III, IV and V.

Inspection of these data will show that the subcutaneous injection of 50 cc. of a 2 per cent solution of sodium hydroxide caused the creatine in the urine of three (Tables II, III, and IV) starving rabbits to completely disappear and in a fourth animal (Table V) creatinuria was notably diminished. In only one instance, Table III, did the urine of the injection day, Jan. 16, become alkaline. A similar injection of alkali on the following day was without influence upon creatine excretion in three out of the four experiments. In the fourth, Table V, creatine excretion continued low. A subsequent (Jan. 19) larger administration of base was without significant influence in two instances, Tables II and V; in one, Table III, there was a noticeable drop in creatine excretion and in Table IV it may be observed that creatinuria was completely abolished. In all cases after the final injections the urines became noticeably alkaline.

The experiments clearly demonstrate that administration of alkali may decrease or entirely inhibit the appearance of creatine in the urine during inanition. This effect cannot be regarded merely as a failure of creatine excretion as, for example, by an action upon kidney function, for under these circumstances it might fairly be assumed that creatinine elimination would also be influenced. The data show that creatinine excretion is not changed materially by administration of alkali. The conclusion is therefore justified that alkali checks or inhibits temporarily the formation of creatine, or, viewed from another standpoint, facilitates its further transformation in the organism. The influence of alkali upon creatine elimination is only temporary, as might be expected, inasmuch as the introduction of alkali merely neutralizes acid produced and does not completely or permanently inhibit acid production. In other words, even though alkali is furnished, acid continues to be formed as the result of the organism's need for fuel. Consequently as the transformation of tissue, in the absence of sufficient glycogen and fat, into energy-producing substances becomes greater and greater the influence of small injections of alkali becomes correspondingly diminished as may be seen from Tables II, III, and V. There seems to be a stage in metabolism where creatine elimination during starvation may be inhibited by administration of alkali. Beyond this point alkali introduction may or may not exert an influence.

TABLE I.

The Influence of Starvation upon the PH *of the Urine and upon Creatine Elimination.*

Date.	Urine.				
	Volume.	Total N.	Creatinine.	Creatine.	PH.
		Rabbit 3.			
1916	*cc.*	*gm.*	*mg.*	*mg.*	
May 1	30	0.56	68	0	7.6
" 2	36	1.04	76	17	5.4
" 3	83	1.71	76	70	4.9
" 4	190	2.58	82	108	4.7
" 5	45	0.34	32	78	5.3
		Rabbit 4.			
May 1	54	0.56	78	0	7.6
" 2	42	0.66	86	20	6.2
" 3	36	0.99	79	55	5.8
" 4	34	0.85	75	95	5.8
" 5	31	1.21	80	90	4.7

TABLE II.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Male rabbit of 2,100 gm.
Jan. 12	60	1.035	Acid.	114	0	Fasting.
“ 13	60	1.040	“	90	29	“
“ 14	50	1.030	“	101	16	“
“ 15	40	1.045	“	72	34	“
“ 16	50	1.045	“	101	0	“ 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
“ 17	60	1.030	“	85	**46**	“
“ 18	50	1.035	Alkaline.	90	42	“
“ 19	80	1.030	“	95	**60**	“ 10.45 a.m. and 5.00 p.m. intravenous injection of 50 cc. 2.0 per cent Na_2CO_3.
“ 20	60	1.030	“	95	**52**	“

TABLE III.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Female rabbit of 2,400 gm.
Jan. 12	250	1.010	Acid.	88	13	Fasting.
“ 13	45	1.035	“	95	14	“
“ 14	50	1.032	“	106	35	“
“ 15	50	1.035	“	100	35	“
“ 16	50	1.035	Alkaline.	101	0	“ 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
“ 17	50	1.035	“	80	**93**	“
“ 18	50	1.040	“	101	71	“
“ 19	80	1.035	“	90	**27**	“ 10.45 a.m. and 5.00 p.m. intravenous injection of 50 cc 2.0 per cent Na_2CO_3.
“ 20	40	1.030	“	83	**29**	“

TABLE IV.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Female rabbit of 2,100 gm.
Jan. 12	270	1.008	Acid.	70	8	Fasting.
" 13	70	1.040	"	81	10	"
" 14	40	1.035	"	75	18	"
" 15	40	1.035	"	75	17	"
" 16	40	1.025	"	59	0	" 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
" 17	50	1.035	Alkaline.	75	23	"
" 18	40	1.030	"	76	37	"
" 19	90	1.030	"	70	23	" 10.45 a.m. and 5.00 p.m. intravenous injection of 50 cc. 2.0 per cent Na_2CO_3.
" 20	30	1.035	"	57	0	"

TABLE V.

The Influence of Alkali upon Creatine Elimination During Inanition.

Date.	Urine.					Remarks.
	Volume.	Specific gravity.	Reaction to litmus.	Creatinine.	Creatine.	
1916	*cc.*			*mg.*	*mg.*	Female rabbit of 2,400 gm.
Jan. 12	170	1.023	Acid.	95	0	Fasting.
" 13	60	1.040	"	101	71	"
" 14	50	1.030	"	85	45	"
" 15	40	1.050	"	90	39	"
" 16	50	1.040	"	95	7	" 10.45 a.m. subcutaneous injection of 50 cc. 2.0 per cent NaOH.
" 17	80	1.030	Alkaline.	115	10	"
" 18	60	1.035	Acid.	124	65	"
" 19	110	1.030	Alkaline.	89	68	" 10.45 a.m. and 5 00 p.m. intravenous injection of 50 cc. 2.0 per cent Na_2CO_3.
" 20	70	1.035	"	108	63	"

SUMMARY.

Administration of alkali during the earlier days of starvation may greatly diminish or completely abolish the creatinuria of that condition. Later in the period of inanition introduction of alkali may not show as marked an influence upon the existing creatinuria.

The results of these experiments lend support to the hypothesis that there exists a relationship between acidosis and creatine elimination.

STUDIES IN CREATINE METABOLISM.

III. THE INFLUENCE OF ALKALI UPON THE CREATINURIA OF PHLORHIZIN GLYCOSURIA.

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(Received for publication, July 31, 1916.)

Creatinuria occurs during phlorhizin glycosuria. Coincident with this there is evidence of carbohydrate deficiency and a well marked acidosis. In view of the fact that in starvation control of the acidosis may lead to disappearance of creatine from the urine of the rabbit it was deemed of importance to determine what influence is to be exerted upon the creatinuria provoked by phlorhizin upon administration of relatively large quantities of sodium bicarbonate.

Methods.

Throughout the period of investigation the dogs employed were allowed to fast. The animals were given subcutaneous doses of 1 gm. of phlorhizin dissolved in 10 cc. of olive oil on 2 successive days previous to the experimental period and thereafter 1 gm. every alternate day. Each day was divided into two 6 hour periods and one 12 hour period, by catheterization. For the estimation of sugar the method of Benedict[1] was employed and creatine was determined according to the procedure of Graham and Poulton.[2] Sodium bicarbonate was administered by mouth in gelatin capsules during the 6 hour periods but was not given during the 12 hour periods.

It is apparent from the data presented in Tables I and II that *the administration of relatively large quantities of sodium bicar-*

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[1] Benedict, S. R., *J. Biol. Chem.*, 1911, ix, 57.

[2] Graham, G., and Poulton, E. P., *Proc. Roy. Soc.*, *Series B*, 1914, lxxxvii, 205.

bonate is without appreciable influence upon the elimination of creatine during phlorhizin glycosuria. This is equally true whether the animal is in a state of complete phlorhizination or not. The preceding paper has shown that administration of alkali is much more potent in abolishing creatinuria in the early days of starvation than it is in the later periods of inanition, perhaps because of the greater difficulty of controlling the condition of acidosis. In the present work it is possible that the same reasoning may hold also, that is, that during phlorhizin intoxication the occasion for acid production is so great that its successful control is improbable. In line with this view stands the fact that the ingestion of alkali as carried through in these experiments seemed to have little or no influence upon the excretion of ketone substances judged by qualitative tests. On the other hand, it would seem reasonable to assume that the giving of these large quantities of alkali should exert at least a slight action upon creatine excretion if the condition of acidosis, that is, alkali depletion, is to be considered as the stimulus for the production of creatine. One is therefore forced to the conclusion that *more than one factor may govern the mechanism leading to creatinuria.*

TABLE I.

The Influence of Alkali upon Creatine Excretion in Phlorhizin Glycosuria.

Date.	Urine.										Remarks.
	Total N.		Dextrose.		Creatinine N.		Creatine N.		D: N.		
	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	
1916	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*			
June 6	1.99	4.85	20.82	34.55	45	100	85	19	10.25	7.12	June 4 and 5 each, 1 gm. phlorhizin.
" 7	1.18		19.83		46		78		16.80		1 gm. phlorhizin. 4 " $NaHCO_3$. per hr.
	1.73	2.91	14.51	34.34	57	103	21	99	8.40	11.78	
		5.46		21.24		63		110		3.90	No $NaHCO_3$.
" 8	1.88		14.22		39		50		7.56		2 gm. $NaHCO_3$ per hr.
	1.96	3.84	13.60	27.82	49	88	45	95	6.95	7.24	
		3.02		6.80		59		100		2.25	No $NaHCO_3$.
" 9	2.80		9.80		34		54		3.50		1 gm. phlorhizin. 2 gm. $NaHCO_3$. per hr.
	1.51	4.31	20.50	30.30	31	65	61	105	13.60	7.02	
		2.07		7.90		42		129		3.82	No $NaHCO_3$.

Urines obtained during periods of alkali administration were strongly alkaline. During the other periods urines were acid.

TABLE II.

The Influence of Alkali upon Creatine Excretion in Phlorhizin Glycosuria.

Date.	Urine.										Remarks.
	Total N.		Dextrose.		Creatinine N.		Creatine N.		D: N.		
	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	6 hr. period.	12 hr. period.	
1916	*gm.*	*gm.*	*gm.*	*gm.*	*mg.*	*mg*	*mg.*	*mg.*			
May 29	1.32		5.98		22		36		4.52		May 27 and 28, 1 gm. phlorhizin.
	1.43	2.75	5.55	11.53	20	42	40	76	3.85	4.20	May 29, no $NaHCO_3$.
" 30		2.27		7.87		26		90		3.47	No Na_2CO_3.
	1.60		6.12		17		49		3.82		1 gm. phlorhizin. 1 " $NaHCO_3$ per hr.
	1.57	3.17	5.68	11.80	15	32	50	99	3.62	3.72	
" 31		2.20		10.30		29		102		4.70	No $NaHCO_3$.
	0.98		3.56		12		51		3.62		2 gm. $NaHCO_3$ per hr.
	1.07	2.05	3.41	6.97	12	24	54	105	3.18	3.40	
June 1		1.48		6.62		22		83		4.48	No Na_2CO_3.
	0.92		2.64		10		36		2.86		1 gm. phlorhizin. 2 " $NaHCO_3$ per hr.
	0.67	1.59	2.00	4.64	8	18	27	63	2.98	2.92	

Urines obtained during periods of alkali administration were alkaline. During the other periods urines were acid.

STUDIES IN CREATINE METABOLISM.

IV. THE RELATIONSHIP OF CREATINURIA TO CARBOHYDRATE METABOLISM AND ACIDOSIS.

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(Received for publication, July 31, 1916.)

In 1908 it was shown by Underhill and Kleiner[1] that the subcutaneous injection of hydrazine sulfate called forth, among other symptoms, a condition of marked creatinuria. The creatine eliminated under these circumstances was much greater than could be accounted for by the attendant starvation. Later work[2] demonstrated that hydrazine injections resulted invariably, in dogs, and occasionally in rabbits, in a state of distinct hypoglycemia without the appearance in the urine of any reducing substance and with a temporary disappearance of glycogen from the liver and muscles, accompanied by an increased respiratory quotient.[3] Recently MacAdam[4] has called attention to the fact that hypoglycemia may be produced invariably in rabbits provided the dose of hydrazine is sufficiently large. The same investigator has also pointed out that there is a close association between the condition of hypoglycemia and creatinuria for "after hydrazine injection in rabbits (a) if no alteration in the sugar content of the blood occurs, no marked creatinuria results; (b) but the excretion of creatine is associated with a condition of hypoglycemia." From these results it would appear that support is afforded the current view concerning creatinuria; namely, that creatine in the urine is closely associated with a deficiency of carbohydrate in the body.

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[1] Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 165.

[2] Underhill, F. P., *J. Biol. Chem.*, 1911, x, 159; also Underhill, F. P., and Hogan, A. G., *ibid.*, 1915, xx, 203.

[3] Underhill, F. P., and Murlin, J. R., *J. Biol. Chem.*, 1915, xxii, 499.

[4] MacAdam, W., *Biochem. J.*, 1915, ix, 229.

As outlined in the preceding papers[5] creatine may find its way into the urine of the rabbit at least under circumstances which exclude the possibility of carbohydrate deficiency. On the other hand, the data presented in the papers to which reference is made indicate that creatinuria is connected with a condition of acidosis in the sense of withdrawal of base from the body. Acidosis and carbohydrate deficiency usually accompany one another, indeed the first may many times be regarded as the sequel of the latter. In hydrazine one has an excellent example of carbohydrate deficiency. The question therefore naturally arises, whether the creatinuria induced by hydrazine is caused by carbohydrate depletion or by an accompanying acidosis. Or must it be recognized that creatine in the urine may arise as a result of two entirely different types of mechanisms being set in motion? May creatine appear in the urine with deficiency of carbohydrate without acidosis and may creatinuria be present in conditions of acidosis with a sufficiency of carbohydrate in the body? The latter part of the question has already been answered in the affirmative by the data in the preceding papers.[5] The query whether creatine may be excreted in the urine under circumstances where carbohydrate depletion prevails without an accompanying acidosis demands an answer which it is the object of the present communication to furnish and for this purpose the creatinuria induced by hydrazine has been studied in the dog.

Methods.

Inasmuch as animals refuse to eat after hydrazine administration it becomes essential to determine the influence of starvation upon the factors under discussion before proceeding to a consideration of the effect of hydrazine. Accordingly dogs were allowed to starve for a period of 5 days, urine being collected in 24 hour periods by catheterization, precautions being used to prevent bladder infection. With these specimens of urine determination was made of the total nitrogen, the creatinine[6] nitrogen, and the creatine[7] nitrogen. The sugar content of the blood drawn from the ear vein was estimated by the method of

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[5] Underhill, *J. Biol. Chem.*, 1916, xxvi, 127, 141, 147.

[6] Folin's method.

[7] Benedict and Myers' method.

Benedict and Lewis.[8] Following the period of inanition the dogs were well fed on a mixed diet over a period sufficient to allow them to regain their normal body weight. At this time they were given subcutaneous injections of hydrazine sulfate in the dosage of 50 mg. per kilo body weight and estimations were made upon the urine and blood as detailed above, except that 12 hour periods were employed instead of 24 hour periods. This was done especially for the purpose of correlating more closely changes in blood sugar and creatine of the urine. Where determinations of the hydrogen ion concentration of the urine were carried through the procedure of Henderson and Palmer[9] was employed.

The data in Tables I, II, III, and IV show very clearly that hydrazine induces a marked creatinuria which is far in excess of that provoked by starvation. The appearance of creatine in the urine in significant amounts is coincident with the period of hypoglycemia. With the return of blood sugar to normal creatinuria falls in general, in one instance to the starvation level. It is evident then that creatinuria and hypoglycemia are closely associated and it is probable that the appearance in the urine of creatine is dependent upon the hypoglycemia. It is also to be noted that the total nitrogen of the urine during the hydrazine period is markedly higher than with simple inanition. From this one might query whether the creatine was derived from tissue disintegration induced by the condition of hypoglycemia.

Hypoglycemia after hydrazine intoxication is not associated with the appearance in the urine of any of the typical compounds usually connected with the term acidosis, as ketone substances, lactic acid, etc. This has been demonstrated by Underhill and Kleiner[1] and by MacAdam.[4] It became of interest therefore to determine the hydrogen ion concentration of the urine as an index to the changes taking place in the body with regard to the acid-base equilibrium. In the dog this is exceedingly difficult of accomplishment where catheterization is practiced, owing to the fact that error is likely to be introduced in the interpretation of the results. For example, if the urine showed a change from an acid to an alkaline reaction it would be difficult to determine convincingly whether the change had occurred in the bladder or elsewhere. Again, in order to insure freedom from bladder infection it is customary in this laboratory to allow a few cc. of boric acid to remain in the bladder after catheterization. It became necessary, therefore, to determine the hydrogen ion concentration upon samples which had been voided spontaneously. The results of such a study first during a preliminary starvation period and then after hydrazine administration are given in Table V. In general, during the period of inanition the urine became progressively more acid *whereas in the hydrazine period the distinctly acid urine voided at the beginning of the period showed a marked temporary change to alkalinity* followed by a resumption of the acidity. The decrease in hydrogen ion concentration corresponds well with the period of low blood sugar as determined by previous investigations since hypoglycemia obtains first from the 36th to the 48th hour after administration of hydrazine and persists for less than 48 hours. The alkalinity of the urine was undoubtedly due to the presence of a carbonate since on acidification carbon dioxide was given off.

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[8] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[9] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393.

These results make it evident that in hydrazine intoxication the increased creatinuria cannot be ascribed to a condition of marked acidosis. Taken in connection with the data already reported they also lead to the conclusion that in the consideration of the cause of creatinuria at least two types of factors may be potent. In the first place creatine may appear in the urine in conditions of acidosis where the question of carbohydrate deficiency is not involved, and secondly, a marked creatinuria may be induced during carbohydrate deficiency even when acidosis is absent. A glance at Tables I to IV will reveal one other characteristic effect of hydrazine; namely, a noticeably increased output of nitrogen when comparison is made with corresponding figures for the period of simple starvation. Will this increased nitrogen output indicating tissue disintegration account for the augmented creatine excretion?

The observations here presented repeat once again the relationship existing between a state of alkalosis and blood sugar content. A previous communication[10] has demonstrated that introduction of alkali into rabbits may at times appreciably lower blood sugar content. After thyreoparathyroidectomy[11] in dogs blood sugar may fall markedly and at this period a condition of alkalosis[12] exists. With hydrazine hypoglycemia it is probable that the same state is present. It is apparent therefore that *there is a close relationship between blood sugar content and the acid-base equilibrium in the body.*

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[10] Underhill, *J. Biol. Chem.*, 1916, xxv, 463.

[11] Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87.

TABLE I.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine.										Blood sugar.	Remarks.
		Total N.		Creatinine N.		Creatine N.		Creatinine N.		Creatine N.			
		12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.		
						Starvation period.							
1916	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
May 2	7.5		4.29		43		3		1.0		0.07	0.096	Bitch of 7.5 kilos. Last meal 24 hrs. previous to beginning of experiment.
" 3	7.2		1.96		44		3		2.0		0.15	0.096	
" 4	7.2		2.40		36		4		1.5		0.17	0.103	
" 5	7.1		2.22		44		3		2.0		0.14	0.102	
" 6	6.9		2.22		43		4		2.0		0.18	0.094	
						Hydrazine period.							
May 15	7.7	0.53		8		12		1.5		2.3		0.107	Animal well fed on mixed diet from May 7 to 14.
		1.42	1.95	20	28	14	26	1.4	1.4	1.0	1.3	0.123	
" 16	7.2	1.40		20		23		1.4		1.6		0.113	May 15, 9 a.m., subcutaneous injection of hydrazine sulfate, 50 mg. per kilo. No food after injection.
		1.98	3.38	25	45	39	62	1.3	1.3	2.0	1.8	0.135	
" 17	7.1	2.05		28		33		1.4		1.6		0.078	
		1.88	3.93	29	57	35	68	1.5	1.4	1.9	1.7	0.109	
" 18	7.0	2.01		23		35		1.2		1.7		0.107	
		1.06	3.07	20	43	38	73	1.9	1.4	3.6	2.4		
" 19	6.8	0.99		18		20		1.8		2.0			
		0.85	1.84	19	37	11	31	2.2	2.0	1.3	1.7	0.100	

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[12] Wilson, D. W., Stearns, T., and Thurlow, M. D., *J. Biol. Chem.*, 1915, xxiii, 100. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *ibid.*, 1915, xviii, 123.

TABLE II.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine.												Blood sugar.	Remarks.
		Total N.		Creatinine N.		Creatine N.		Creatinine N.		Creatine N.					
		12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.				
Starvation period.															
1915	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*			*per cent*	
Nov. 8	16.0		5.22		118		16		2.2		0.3			0.15	Bitch of 16 kilos. Last meal 24 hrs. previous to beginning of experiment.
" 9	15.1		3.82		196		7		5.1		0.2			0.14	
" 10	14.8		2.98		205		5		6.8		0.1			0.13	
" 11	14.6		2.48		174		10		7.0		0.4			0.14	
" 12	14.4		2.25		154		37		6.8		1.6			0.11	
Hydrazine period.															
Dec. 6	15.1	2.28		78		8		3.4		0.3				0.15	Animal well fed on mixed diet from Nov. 13 to Dec. 5. Dec. 6, 9 a.m., subcutaneous injection of hydrazine sulfate, 50 mg. per kilo.
		3.14	5.42	80	158	17	25	2.5	2.9	0.5	0.4			0.12	
" 7	14.2	3.47		89		78		2.5		2.2				0.06	
		4.23	7.70	102	191	98	176	2.4	2.4	2.3	2.2			0.04	
" 8	14.1	4.65		87		49		1.9		1.0				0.11	
		5.16	9.91	77	164	61	110	1.4	1.6	1.1	1.1			0.16	
" 9	13.9	4.70		86		94		1.9		2.0				0.11	
		3.66	8.36	64	150	122	216	1.7	1.8	3.3	2.5			0.12	
" 10	13.5	4.55		155		86		3.4		1.9				0.12	

TABLE III.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine.										Blood sugar.	Remarks.
		Total N.		Creatinine N.		Creatine N.		Creatinine N.		Creatine N.			
		12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.	12 hr. period.	24 hr. period.		
Starvation period.													
1915	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Nov. 16	6.2		2.10		56		0		2.6		0.1	0.11	Bitch of 6.2 kilos.
" 17	6.1		1.53		50		7		3.2		0.4	0.12	Last meal 24
" 18	6.0		1.40		61		20		4.3		0.1	0.13	hrs. previous
" 19	5.9		1.34		56		7		4.1		0.05	0.12	to beginning of
" 20	5.7		1.39		58		17		4.1		0.1	0.11	experiment.
Hydrazine period.													
1916													
Jan. 31	6.3	1.33		25		0		1.8		0		0.12	Animal well fed
		1.89	3.22	24	49	9	9	1.2	1.5	0.4	0.2	0.13	on mixed diet
Feb. 1	5.8	1.67		22		14		1.3		0.8		0.12	from Nov. 21
		2.26	3.93	25	47	27	41	1.1	1.2	1.2	1.0	0.08	until Jan. 30.
" 2	5.7	2.00		25		24		1.2		1.1		0.03	Jan. 31, 9 a.m.
		2.10	4.10	27	52	22	46	1.2	1.2	1.0	1.1	0.04	subcutaneous
" 3	5.7	1.68		26		28		1.5		1.7		0.13	injection of
		1.44	3.12	24	50	9	37	1.6	1.6	0.6	1.2	0.12	hydrazine sul-
" 4	5.6	1.28		23		3		1.8		0.2		0.12	fate, 50 mg. per
		1.15	2.43	26	49	1	4	2.2	2.0	0.1	0.1	0.12	kilo.
" 5	5.5	1.05		25	49	0.6		2.3		0.05			

TABLE IV.

The Influence of Hydrazine upon the Excretion of Creatine.

Date.	Body weight.	Urine. Total N. 12 hr. period.	Urine. Total N. 24 hr. period.	Urine. Creatinine N. 12 hr. period.	Urine. Creatinine N. 24 hr. period.	Urine. Creatine N. 12 hr. period.	Urine. Creatine N. 24 hr. period.	Urine. Creatinine N. 12 hr. period.	Urine. Creatinine N. 24 hr. period.	Urine. Creatine N. 12 hr. period.	Urine. Creatine N. 24 hr. period.	Blood sugar.	Remarks.
Starvation period.													
1916	*kg.*	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
May 2	6.4		2.16		40		0		1.9		0	0.087	Bitch of 6.4 kilos. Last meal 24 hrs. previous to beginning of experiment.
" 3	6.2		2.88		44		2		1.5		0.07	0.088	
" 4	6.0		1.82		50		12		2.7		0.66	0.094	
" 5	5.9		1.77		53		17		3.0		0.96	0.092	
" 6	5.8		1.90		53		15		2.8		0.80	0.089	
Hydrazine period.													
May 15	6.6	1.09		18		3		1.6		0.28		0.11	Animal well fed on mixed diet from May 7 to 14. Last meal on May 14. May 15, 9 a.m., subcutaneous injection of hydrazine sulfate, 50 mg. per kilo. No food after injection.
		1.49	2.58	20	38	11	14	1.3	1.5	0.74	0.5		
" 16	6.1	1.10		18		12		1.6		1.1		0.126	
		1.20	2.30	18	36	42	54	1.5	1.6	3.5	2.3	0.140	
" 17	5.9	1.51		23		37		1.5		2.4		0.079	
		1.55	3.06	24	47	36	73	1.5	1.5	2.3	2.4	0.102	
" 18	5.8	1.45		18		28		1.2		1.9		0.099	
		0.98	2.43	16	34	35	63	1.6	1.4	3.6	2.6		
" 19	5.8	0.89		24		29		2.7		3.3		0.098	
		0.84	1.73	20	44	4	33	2.4	2.5	0.48	1.9		
" 20	5.8	0.83		18		12		2.2		1.4		0.098	

TABLE V.

The Influence of Starvation and of Hydrazine upon the Hydrogen Ion Concentration of the Urine.

Date.	Dog 9.		Dog 10.	
	Time of micturition.	P_H.	Time of micturition.	P_H.
Starvation period. Last meal May 8, 10 a.m.				
May 8	6 p.m.	7.14		
" 9	9 a.m.	7.00	3 p.m.	7.40
" 10	9 "	7.38		
	5 p.m.	7.00		
" 11	9 a.m.	6.90	9 a.m.	7.00
	5 p.m.	6.90		
" 12	9 a.m.	6.30		
" 13			9 "	6.00
Hydrazine period. May 25, subcutaneous injection of 50 mg. per kilo hydrazine sulfate.				
May 26	9 a.m.	5.70	9 a.m.	6.15
" 27	10 "	5.70	12 m.	6.30
	5 p.m.	6.70	5 p.m.	6.30
" 28	9 a.m.	7.41	10 a.m.	9.27
	3 p.m.	7.45		
	9 "	6.70		
" 29	9 a.m.	5.50	9 a.m.	7.38
			9 p.m.	6.00
" 30			9 a.m.	5.70

SUMMARY.

The subcutaneous administration of hydrazine to dogs induces a marked creatinuria which in general closely parallels the period of hypoglycemia provoked. These observations corroborate the results of previous investigations.

During the period of hypoglycemia and most marked creatinuria the hydrogen ion concentration of the urine is greatly depressed—even to the point of marked alkalinity. The alkalinity is probably due to the presence of carbonates.

The relationship of hypoglycemia and alkalosis again emphasizes the significance of acid-base equilibrium in the regulation of the blood sugar content.

The results here presented make it evident that creati elimination in the urine may be induced by at least two diffe ent sets of conditions (1) creatine may appear in the urine states of acidosis where carbohydrate deficiency is not involv and (2) creatinuria may be present during carbohydrate de ciency even in the absence of acidosis.

ALTERATIONS IN THE OUTPUT OF CERTAIN URINARY CONSTITUENTS AS DETERMINED BY CHANGES IN THE CHARACTER OF THE DIET.

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Changes in the character of the diet may induce the appearance of creatine in the urine or may cause it to disappear therefrom. Rabbits fed upon a mixture of corn and oats excrete creatine in the urine. A carrot diet causes the urinary creatine to disappear. The character of the protein of the diet plays little or no rôle since the urine eliminated by rabbits upon a mixture of corn, oats, and carrots remains creatine-free. By the addition of hydrochloric acid to the mixed diet creatinuria is induced. Since corn and oats are acid-producing foods and carrots are base producers, the facts noted above with respect to the appearance or abolishment of creatinuria have been explained by the hypothesis that a condition of acidosis, in the sense of base withdrawal from the body, is responsible for the appearance of creatine in the urine. This condition may be abolished or prevented by the ingestion of base-producing foods, like carrots.

In view of these facts it seemed desirable to determine whether changing the character of the diet in the manner indicated above would effect alterations in the output of other urinary constituents, consideration of course being given to the differences in intake of various components of the diet. This attempt forms the subject of the present paper, the results obtained being detailed in the tables appended.

Methods.

The urine was divided into 24 hour periods by expressing it from the bladder by pressure through the abdominal wall. Total nitrogen in the urine was estimated by the Kjeldahl method; creatinine by the Folin method; creatine according to Benedict and Myers; hydrogen ion concentration by the procedure of Henderson and Palmer; ammonia by the Folin aeration method; phosphorus by titration with uranium nitrate; and calcium according to McCrudden. Phosphorus in the food was estimated by the Neumann method. The "grain diet" consisted of 50 gm. cracked corn and 50 gm. oats. The "carrot diet" contained 500 gm. fresh carrots. The "mixed diet" consisted of 25 gm. cracked corn, 25 gm. oats, and 250 gm. carrots. During the acid period, hydrochloric acid was administered by stomach tube twice daily at intervals of 8 hours in the dosage of 50 cc. 0.25 N HCl. The variations in the intake of nitrogen and phosphorus on the grain diet are due to the variable amounts of food actually ingested.

The one striking feature of the experiments may be seen by inspection of the tables relative to the grain diet. Compared with the intake *the urinary phosphorus output* is very large. During no other period did this condition obtain. Inasmuch as at this interval the hydrogen ion concentration of the urine was greatest it is probable that the excessive elimination of phosphorus in this instance at least may be accepted as a means through which the rabbit regulates the acid-base equilibrium of the organism. Ammonia plays little rôle here apparently for in no case was there a significant excretion of this constituent during the grain period. On the other hand, the elimination of ammonia in the rabbit is very difficult of interpretation for although the ammonia output in Rabbits 2 and 3 was negligible throughout irrespective of the character of the diet significant quantities of ammonia were excreted through the kidney by Rabbit 1, even though a base-producing diet was fed. The output was greatest when hydrochloric acid was administered although not much greater than that observed without added acid. With Rabbit 4 ammonia excretion was markedly increased only during the period of hydrochloric acid feeding. It is probable, then, that ammonia may play a part in the acid-base equilibrium of the body under certain conditions but the exact circumstances which allow or prevent this type of regulation in this manner remain to be determined.

Urinary calcium output seems to be so variable under the experimental conditions that no conclusion regarding the significance of this element seems justified.

There is the same parallelism between creatine output and hydrogen ion concentration of the urine observed in previous papers from this laboratory upon the subject.

The experiments demonstrate beyond doubt that the excretion of creatine during the grain diet cannot be explained by insufficiency of food intake since in all instances the animals were undoubtedly in nitrogen equilibrium as indicated by the probable positive nitrogen balance. During the carrot diet, on the other hand, a negative nitrogen balance is always in order; but creatinuria does not develop, hence it is evident that the creatinuria observed upon the grain diet cannot be explained by food insufficiency.

The tables follow.

TABLE I.

Rabbit 1, Male, 2,360 Gm., Weight April 14. 2,330 Gm.

Date.	Diet.	Intake (food).		Urine.						
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.
1916		*mg.*	*mg.*	*cc.*	*1.0–*	*mg.*	*mg.*	*mg.*	*mg.*	
Mar. 30	Mixed.	1,115	180	145	20	684	4	77	0	9.2
" 31	Grain.	1,060	84	60	32	920	0	147	27	4.7
April 1	"	1,060	84	43	43	806	0	124	37	4.9
" 2	"	1,060	84	40	48	786	0	115	56	4.9
" 3	"	1,364	108	34	60	830	5	104	38	4.9
" 4	"	1,288	102	33	55	890	0	97	44	4.9
" 5	"	954	76	33	50	870	4	81	44	5.3
Average		1,131	89	41	1.044	850	1	111	41	4.9
April 6	Carrot.	715	240	160	20	830	5	81	34	9.2
" 7	"	715	240	385	14	1,102	20	108	0	9.2
" 8	"	715	240	354	16	1,092	13	90	0	8.0
Average		715	240	299	1.016	1,008	12	93	11	8.8
April 9	Mixed.	1,115	180	160	20	699	31	68	0	8.7
" 10	"	1,115	180	150	21	783	38	85	0	8.7
Average		1,115	180	155	1.020	741	34	76	0	8.7
April 11	Mixed +HCl.	1,115	180	238	11	739	49	84	48	8.0
" 12	"	1,115	180	240	11	727	40	91	51	7.3
" 13	"	1,115	180	190	30	658	31	95	28	6.9
Average		1,115	180	222	1.017	702	40	90	42	7.4
April 14	Carrot.	715	240	300	21	776	18	122	0	9.2
" 15	"	715	240	390	14	1,176	45	120	0	9.2
" 16	"	715	240	332	14	1,155	14	102	0	9.2
Average		715	240	340	1.016	1,035	25	114	0	9.2

TABLE II.

Rabbit 2, Male, 3,420 Gm., Weight April 14, 3,100 Gm.

Date.	Diet.	Intake (food).		Urine.								
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.	Phosphorus.	Calcium.
1916		*mg.*	*mg.*	*cc.*	*1.0–*	*mg.*	*mg.*	*mg.*	*mg.*		*mg.*	*mg.*
Mar. 30	Mixed.	1,115	180	290	17	1,379	0	162	0	9.27	188	32
" 31	Grain.	1,060	84	93	20	699	0	95	0	7.48	155	28
April 1	"	1,060	84	47	20	386	0	70	0	6.00	85	7
" 2	"	1,060	84	190	22	1,675	3	190	21	5.70	303	18
" 3	"	1,060	84	80	17	673	6	97	13	6.00	125	20
" 4	"	1,060	84	142	16	1,159	0	122	53	5.70	213	27
" 5	"	833	66	110	13	913	0	119	50	5.70	163	22
Average		1,022	81	110	1.018	917	1	115	23	6.09	174	20
April 6	Carrot.	715	240	285	10	1,381	15	142	0	8.70	118	27
" 7	"	715	240	394	12	1,142	0	95	0	9.27	76	15
" 8	"	715	240	510	15	1,679	0	146	0	8.00	124	66
Average		715	240	396	1.012	1,400	5	127	0	8.65	106	36
April 9	Mixed.	1,115	180	214	20	1,000	0	106	0	8.70	59	61
" 10	"	1,115	180	156	18	843	0	108	0	8.70	89	49
Average		1,115	180	185	1.019	921	0	107	0	8.70	74	55
April 11	Mixed +HCl.	1,115	180	328	12	1,513	0	187	0	8.00	179	31
" 12	"	1,115	180	290	12	1,218	3	130	56	4.90	188	62
" 13	"	1,115	180	174	37	1,166	3	125	59	5.70	168	56
Average		1,115	180	264	1.020	1,299	2	147	38	6.20	178	49

TABLE III.

Rabbit 3, Male, 2,320 Gm., Weight April 11, 2,280 Gm.

Date.	Diet.	Intake (food).		Urine.						
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.
1916		*mg.*	*mg.*	*cc.*	*1.0—*	*mg.*	*mg.*	*mg.*	*mg.*	
Mar. 30	Mixed.	1,115	180	145	20	583	8	85	0	9.27
" 31	Grain.	1,060	84	60	26	684	0	101	0	8.00
April 1	"	1,060	84	53	33	769	3	108	8	6.00
" 2	"	1,060	84	54	34	806	0	101	8	5.70
" 3	"	909	72	59	30	811	0	113	14	5.70
" 4	"	909	72	63	23	874	1	84	23	5.70
" 5	"	682	54	64	27	850	0	81	39	5.70
Average		946	75	59	1.029	799	0.6	98	15	6.13
April 6	Carrot.	715	240	174	17	1,037	1	96	0	8.70
" 7	"	715	240	350	15	1,143	0	108	0	8.70
" 8	"	715	240	326	17	723	0	100	0	8.00
Average		715	240	283	1.018	967	0.3	101	0	8.46
April 9	Mixed.	1,115	180	190	17	729	0	90	0	8.00
" 10	"	1,115	180	172	17	611	0	101	0	8.00
Average		1,115	180	180	1.017	820	0	95	0	8.00

TABLE IV.

Rabbit 4, Male, 1,980 Gm., Weight April 11, 2,060 Gm.

Date.	Diet.	Intake (food).		Urine.								
		Nitrogen.	Phosphorus.	Volume.	Specific gravity.	Total N.	NH_3 N.	Creatinine.	Creatine.	P_H.	Phosphorus.	Calcium.
1916		mg.	mg.	cc.	1.0–	mg.	mg.	mg.	mg.		mg.	mg.
Mar. 30	Mixed.	1,115	180	155	18	546	0	82	0	9.27	81	26
" 31	Grain.	1,060	84	45	35	768	0	90	0	8.70	134	10
April 1	"	1,060	84	28	50	454	0	81	0	6.30	99	14
" 2	"	1,060	84	25	55	543	4	95	6	6.00	109	29
" 3	"	1,060	84	40	47	695	0	73	16	5.30	140	7
" 4	"	757	60	40	47	763	2	61	22	5.70	157	28
" 5	"	757	60	41	43	662	0	62	22	5.70	138	42
Average		959	76	36	1.046	647	1	77	11	6.28	129	21
April 6	Carrot.	715	240	270	12	1,010	5	101	13	9.27	80	23
" 7	"	715	240	402	13	985	3	86	0	8.70	95	24
" 8	"	715	240	380	15	759	0	73	0	8.70	72	19
Average		715	240	350	1.013	918	3	87	4	8.89	82	22
April 9	Mixed.	1,115	180	158	20	736	5	75	0	8.70	76	20
" 10	"	1,115	180	158	19	638	7	81	0	8.70	76	46
Average		1,115	180	158	1.019	687	6	78	0	8.70	76	33
April 11	Mixed	1,115	180	262	12	794	12	109	0	7.48	115	37
" 12	+HCl.	1,115	180	206	21	1,108	25	67	73	5.30	141	48
Average		1,115	180	234	1.016	951	18	88	36	6.39	128	42

SUMMARY.

Upon a diet of corn and oats the output of phosphorus in the urine of rabbits is far in excess of the intake of this element in the food. Since the hydrogen ion concentration of the urine is very high under these circumstances the great excess of phosphoric acid in the urine may be regarded as a method of regulating acid-base equilibrium in the body of the rabbit.

Ammonia excretion is variable under changing conditions of diet, and although it appears that this base may function as a neutralizing agent at times, further experiments are necessary before the laws of ammonia excretion in the rabbit can be formulated.

Calcium elimination is too variable to justify inferences being drawn under the experimental conditions.

The results obtained indicate that the creatinuria observed upon a grain diet cannot be explained upon the hypothesis of food insufficiency.

THE INTERRELATIONS OF BLOOD FAT AND BLOOD SUGAR CONTENT OF DOGS UNDER THE INFLUENCE OF HYDRAZINE.

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The metabolism of carbohydrate and that of fat are closely associated and interdependent. This relationship is more evident under conditions of abnormal metabolic activity than it is when the nutritive rhythm remains undisturbed. The most striking instances of the interrelationships of fat and carbohydrate in metabolism are to be observed in the pathological states induced by intoxication with phosphorus, phlorhizin, and hydrazine, and in alcoholism and diabetes, which are characterized by the so called "fatty" livers.[1] In general a "fatty" liver is associated with a deficiency of carbohydrate in that organ. The "sugar-hungry cells" attract fat to themselves in much greater quantity than they can burn it.[2] This implies transport of fat through the blood stream. Under ordinary nutritive circumstances the fat content of dogs is quite constant for the individual and for the species, which indicates an efficient regulation.[3] The blood fat content therefore may be regarded as one of the body constants like the blood sugar content; and like it is subject to deviation by a variety of agencies. The factors which are potent in causing changes in blood fat content have not been sufficiently studied to correlate them with those which are effective in producing alterations in blood sugar content.

It has been demonstrated repeatedly that administration of hydrazine to dogs causes low blood sugar content, loss of glycogen from both the liver and muscles, and induces a "fatty" liver. The "fatty" liver presupposes fat infiltration with a consequent transport through the blood stream. Is the "fatty" liver produced by the storage of fat from the blood, the amount of fat in the latter remaining unchanged, or is there an actual increase of the fat of the blood during hydrazine intoxication? If blood fat is augmented under these circumstances does it bear any relation to blood sugar content? These are questions which seem of fundamental importance in a consideration of the mechanism of blood fat regulation. They form the subject of the present investigation.

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[1] For a discussion of the subject see Rosenfeld. *Ergebn. Physiol.*, 1902, i. 651; 1903, ii, 50.

[2] Mandel, A. R., and Lusk, G., *Am. J. Physiol.*, 1906, xvi, 129.

[3] Bloor, W. R., *J. Biol. Chem.*, 1914, xix, 1.

Methods.

Dogs under the influence of hydrazine refuse food or fail to retain it. Hence a fasting condition is inevitable in hydrazine experimentation. Results obtained by the action of this poison should therefore be interpreted with respect to the effects produced by fasting alone. Accordingly dogs were fasted for periods of 6 days, determinations of the blood fat and blood sugar being made daily upon samples obtained from a marginal ear vein. Blood fat was estimated by the method of Bloor with a Richards nephelometer. Blood sugar content was determined in part by the procedure of Forschbach and Severin and in part by that of Benedict and Lewis. Subsequent to the fasting period the animals were well fed with mixed diets for varying intervals and were then given subcutaneous injections of hydrazine sulfate in the dosage of 50 mg. per kilo body weight. Blood fat and blood sugar content were then determined as in the fasting period except that the estimations were carried through at 12 hour intervals.

The results obtained with five dogs are presented in the table During the fasting period there is a general tendency for blood fat content to diminish in the first days of inanition. This decrease is followed by an augmentation of the blood fat somewhat beyond the initial value. The outcome of these experiments is somewhat different from that of Bloor who found either

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[4] Bloor, *J. Biol. Chem.*, 1915, xxiii, 317.

The Relationship of Blood Sugar to Blood Fat in Starvation and in Hydrazine Poisoning.

Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.	Date.	Body weight.	Blood sugar.	Blood fat.
Dog 3.	Starvation period.			Dog 4.	Starvation period.			Dog 1.	Starvation period.			Dog 2.	Starvation period.			Dog 5.	Starvation period.		
1915	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*	*1915*	*kg.*	*per cent*	*per cent*
Nov. 29	11.1	*0.13	*1.01	Nov. 16	6.3	*0.12	*0.91	Nov. 29	9.3	*0.13	*0.71	Nov. 16	6.3	*0.12	*0.91	Nov. 8	16.0	*0.15	*0.69
" 30	11.0	0.11	0.84	" 17	6.2	0.11	0.81	" 30	9.2	0.17	0.69	" 17	6.2	0.11	0.81	" 9	15.6	0.14	0.72
Dec. 1	10.8	0.16	0.84	" 18	6.0	0.12	0.77	Dec. 1	9.0	0.12	0.67	" 18	6.0	0.12	0.77	" 10	15.1	0.13	0.74
" 2	10.6	0.13	0.90	" 19	5.9	0.13	0.80	" 2	8.9	0.13	0.71	" 19	5.9	0.13	0.80	" 11	14.8	0.14	0.74
" 3	10.4	0.14	1.02	" 20	5.8	0.12	0.84	" 3	8.8	0.12	0.80	" 20	5.8	0.12	0.84	" 12	14.6	0.11	0.79
" 4	10.2	0.12	1.05	" 21	5.7	0.11	0.95	" 4	8.6	0.13	0.83	" 21	5.7	0.11	0.95	" 13	14.4	0.12	0.87
Dog 3.	Hydrazine period.			Dog 4.	Hydrazine period.			Dog 1.	Hydrazine period.			Dog 2.	Hydrazine period.			Dog 5.	Hydrazine period.		
1915				*1916*				*1915*				*1915*				*1915*			
Oct. 24	8.2	†0.12	†1.12	Feb. 1	6.3	†0.12	†0.79	Oct. 11	8.0	†0.13	†0.73	Oct. 18	6.6	†0.11	†0.90	Dec. 6	15.1	†0.14	†0.74
		0.16	1.15			0.12	0.81			0.12	0.74			0.13	0.79			0.15	0.84
" 25	7.6	0.18	1.21	" 2	5.8	0.08	0.91	" 12	7.5	0.15	0.82	" 19	6.2	0.12	0.81	" 7	14.2	0.12	1.09
		0.15	1.72			0.03	0.99			0.15	0.90			0.05	0.96			0.06	1.26
" 26	7.5	0.03	2.04	" 3	5.7	0.04	1.13	" 13	7.3	0.08	0.94	" 20	6.2	0.03	1.21	" 8	14.1	0.04	1.40
		0.02	1.90			0.12	1.01			0.06	0.94			0.02	1.30			0.11	1.21
" 27	7.4	0.12	1.71	" 4	5.7	0.13	0.92	" 14	7.1	0.05	1.04	" 21	6.0	0.15	1.11	" 9	13.9	0.16	1.08
		0.13	1.25			0.12	0.95			0.03	0.86			0.11	1.07			0.11	0.93
" 28	7.2	0.11	1.14	" 5	5.6	0.12	0.83	" 15	7.1	0.08	0.79	" 22	5.9	0.12	1.01	" 10	13.7	0.12	0.95
										0.14				0.13	0.95			0.12	0.98
								" 16	6.9	0.13	0.77	" 23	5.8	0.11	0.95				
										0.12	0.81								

* 24 hr. periods.

† 12 hr. periods.

an increase of blood fat at first followed by a decrease, or else there was no increase.[3] Our animals were taken at random but were all in a well fed condition. The agreement in blood fat of the different animals is fairly close and tends to confirm Bloor's statement that blood fat is quite constant for the species. There seems to be little or no relation between blood fat and blood sugar content during the period of starvation studied.

In the hydrazine period *blood fat shows an increase to a maximum which is coincident with the condition of hypoglycemia induced by hydrazine.* As blood sugar content regains the normal, blood fat assumes a value approximating that of the fasting period.

Whether blood sugar content regulates the blood fat or whether both sugar and fat in the blood are controlled by some other mechanism must be determined by future investigation.

SUMMARY.

In the first days of fasting there is a tendency for the blood fat of dogs to be decreased. This period is followed by one in which there is an increase of fat content to approximately the initial value.

There seems to be little or no relation between blood fat and blood sugar content during the interval of inanition studied.

In hydrazine poisoning blood fat is markedly increased, the maximum being coincident with the condition of hypoglycemia characteristic of hydrazine. Blood fat returns to the fasting value as the sugar of the blood regains the normal.

THE NUTRITIVE VALUE OF YEAST, POLISHED RICE, AND WHITE BREAD, AS DETERMINED BY EXPERIMENTS ON MAN.

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Heretofore the vitamines in foods have been disregarded in all nutrition experiments on man.

The experiments performed on four normal men, as described in this paper, were undertaken with the view to determining primarily the food value of a vitamine-free diet as compared with one containing vitamines. It was found, however, that to insure reliable results the experiment should be carried over a much longer period of time than we were able to devote to it, but as metabolism experiments on man fed on a diet of such simple composition, and under such exact conditions, are so unusual this contribution may be of value. A survey of the work of the past 20 years was covered during this experiment, and we find, where the problem of a protein minimum is considered, that there are two distinct theories.

Abderhalden (1) and his school claim that, as the composition of the body, and especially of the blood proteins, is so different from food proteins, the best protein minimum can be obtained by feeding proteins of the same species.

Hindhede (2), on the contrary, was able to show that when using potatoes as practically the only food, 1 gm. of the absorbed potato nitrogen is equivalent to 1 gm. of body nitrogen. The experiments of the latter author are carried over such a long period that they can be regarded as entirely conclusive. One cause of discrepancy of opinions on this question is that the importance of vitamines in metabolism has been overlooked in most of the more recent experiments. It was pointed out some time ago by one of us (3) that when vitamines are absent in the food mixture, all animal organisms supplied with a complete food, especially as regards all the necessary amino-acids, will in spite of this come into a negative balance if the experiment is conducted sufficiently long. Hardly any workers in the field of metabolism have realized the importance of this vitamine factor, and some of the conclusions as to the adequacy of proteins or protein mixtures may be erroneously attributed to the incompleteness of the protein. In numerous metabolism experiments on dogs Abderhalden (1) and his school have disregarded the vitamine factor entirely. They often compare a food containing artificially digested meat and vitamines with a food mixture deficient in these latter ingredients. Hindhede (4), on the contrary, takes this vitamine factor into account and gives a potato diet on which no deficiency diseases occur (5). Studying the earlier literature on protein minimum we find that the work of Munk (6) and of Rosenheim (7) gives a typical illustration of the point we wish to emphasize. Dogs which they used in their experiments were given both rice and meat powder, but they overlooked the fact that the greater part of the vitamines was lost by drying and heating. The authors gradually decreased the meat in the diet and replaced it by more carbohydrates in the form of rice. As the result of this procedure the dogs showed a negative nitrogen balance and some of them died of the effects described by Schaumann (8) when he fed them on extracted meat; the others recovered when fresh meat was added.

We know now through the work of one of us (9) and of Braddon and Cooper (10) that the amount of carbohydrates in the diet cannot be increased indiscriminately, as a certain amount of vitamine can take care of only a limited amount of carbohydrates. In other words, there must be a fixed ratio between the vitamine content and the carbohydrate. If this ratio is destroyed, disordered nutrition of the tissue cell occurs. The conclusions of Munk and Rosenheim that dogs require a large supply of protein are erroneous, as some years ago Funk performed metabolism experiments in which dogs were readily brought into nitrogen equilibrium with meat powder or milk powder, while difficulties were encountered when they were fed on edestin or gliadin. In one of the experiments of Abderhalden and Funk (11), where comparative studies were made as to the value of edestin and gliadin, a great number of the dogs died. In one case a nitrogen equilibrium was established for a short period with gliadin but, notwithstanding, the dog steadily lost weight. We do not wish to minimize the importance of aromatic amino-acids, cystine, trytophane, and lysine, but we wish to point out that many of the experiments on the necessity of certain amino-acids should be repeated by taking the vitamine factor into account.

In studies on protein minimum this latter condition should also receive consideration. In a recent paper of Abderhalden, Fodor, and Röse (12) we find a comparative study of the food value of potatoes as compared with whole bread and white bread. The protein minimum was lowest in the case of potatoes (4.5 gm. nitrogen), higher with whole meal bread (7.0 gm. nitrogen), and still higher with white bread (9.+ gm. nitrogen), a possible reason being that the white bread was deficient in vitamines.[1]

EXPERIMENTAL.

Four of the members of the laboratory staff volunteered their services in the feeding experiments with yeast, white rice, and bread, and continued their duties during the whole experimental period. Our diet before the experiment was of normal variety and quantity, in contrast to former investigations, in which the subjects even before the experiment were accustomed to eat food without change or variety. The attempt was made to live on yeast[2] as the only source of nitrogen. During the preliminary period the amount of nitrogen in the excreta was studied under normal dietary conditions. The necessary amount of calories was then supplied in the form of fruit and vegetables. These were chosen to contain the minimum amount of nitrogen and a still smaller amount of protein nitrogen.

Although yeast has been extensively used for feeding cattle for a number of years, its use for human consumption has been suggested only recently in Germany, owing to the scarcity of meat. As a meat substitute it was strongly recommended by Schottelius (13), who has used it in quantities of 50 to 75 gm. daily in addition to the ordinary diet, and he claims that it is well utilized in

[1] Averages in feces were: On potatoes 0.4 to 0.7 gm. nitrogen, in whole meal bread 1.6 to 1.9, and in the case of white bread 1.5 gm. nitrogen. The lower value of white bread is therefore not due to faulty assimilation.

[2] Yeast used by us was a dried anaerobic yeast preparation which was put at our disposal by the American Pure Yeast Company of New York.

the human body. Before undertaking our experiments we tried the availability of yeast in rats, substituting it for casein nitrogen. The animals, records of which will be published later, show that *young rats can live on yeast as the sole nitrogen source for quite a long while, although it has not yet been proven whether they can subsist on it indefinitely.* The experiment showed that yeast contains a protein apparently complete as to its composition in amino-acids, corroborating Neuberg's work (14) on the proteins of yeast. But there was some difficulty in determining how much of the yeast nitrogen is represented by protein nitrogen. Neuberg, by a complicated method, ascertained that yeast protein somewhat resembles casein, and amounted in Schroeder's yeast to 48.3 per cent of the total nitrogen, whereas the bottom fermentation yeast K, from the Institute for Brewing in Berlin, was found to contain only 46.3 per cent. Bokorny (15) states that yeast in a dried state contains 60 per cent protein. We also attempted to determine the protein content of our yeast and found that the usual method of determination, as used by Stutzer, failed completely. This method showed that about 90 per cent of the yeast nitrogen was protein nitrogen. Finally, we tried to estimate the yeast nitrogen indirectly by determining the purine base nitrogen, and for this determination followed the method of Burian and Schur (16), hydrolyzing the yeast with dilute sulfuric acid and precipitating the purine bases in an alkaline solution, with sodium bisulfite and copper sulfate, then estimating the nitrogen in the copper precipitate. In this way we recovered 13 per cent of the yeast nitrogen as purine bases, which undoubtedly was too low a figure.

The literature records only a few actual metabolism experiments with yeast, one of Praussnitz and Menicanti (17), and another of Thomas (18), who, while experimenting with other foodstuffs, prepared a pressed yeast with butter, and used it for 3 days. The nitrogen balance in this case was negative with over 4 gm. of nitrogen in the form of yeast. The yeast was well absorbed as shown by the feces nitrogen, which was rather low.

The utilization of yeast was insufficient in our experiment where yeast was taken for nearly a fortnight, as the nitrogen content of feces was high, and in some instances undigested yeast was detected. With two out of four persons a practically positive balance was obtained for several days. The experiments were performed in the beginning of June, when cold weather prevailed and some difficulty was experienced in maintaining bodily heat. In spite of the small food supply there was no craving for food. The bowels moved with regularity and no diarrhea occurred. The bad utilization of yeast may be partially due to the fact that the yeast had a disagreeable taste and was unpleasant to eat for any length of time.

During the second period the diet consisted of white rice, white bread, and butter. We had very much less nitrogen in this form than Kumagawa (19), who weighed 49 kg., and took 600 gm. rice, 100 gm. Miso (a fermented mixture of rice and beans), 10 cc. soja, 600 cc. beer, 28 gm. sugar, 300 gm. of turnip-cabbage, and 600 cc. tea, averaging 8.75 gm. nitrogen per day. Our vitamine- and purine-free diet was supplemented in the later period by vitamine from 50 cc. of autolyzed yeast, prepared according to the method of Seidell (20), using Lloyd's reagent, a colloidal aluminum silicate preparation of unknown composition.

In one case which was kept as a control, the vitamine was omitted. We were unable to find that upon its addition the balance became more positive. The reason for this may be ascribed to the shortness of the experiment, or to the fact that the vitamine preparation, made in the above mentioned way, was not sufficiently active.

An added interest in our metabolism experiments was the chemical examination of blood. The negative balance found is partially due to this, and although the nitrogen lost in this way was determined, it was not included in the balance, as the blood nitrogen is partially composed of food nitrogen already assimilated, and partially of nitrogen which would be subsequently eliminated through the kidney or through the intestinal tract. The loss, however, is added in the blood analyses. On the days of the chemical blood examinations, a partition of the different nitrogenous constituents of the urine was also determined. This had been done once before in a metabolism experiment by Folin (21), but in his case the nitrogen intake was held to normal limits (about 16 gm.). In a few instances on the vitamine-free diet the sugar in the urine was determined by Bertrand's method. Although the blood sugar was not more than usual, the urinary sugar was much higher than in normally fed cases, as determined by the same method by Funk (22). This may be due to alimentary glycosuria following a high carbohydrate diet, or may be analogous to the findings of one of us with Schönborn (23) in pigeons, where a considerable increase in blood sugar was found on a rice diet. We shall soon begin to investigate this problem in diabetics on vitamine-free and vitamine-containing diets.

The nitrogen content of foodstuffs consumed by us was determined actually in all instances. The feces at the beginning of the feeding experiment were marked by feeding charcoal. In the second period this was not necessary, as the color changed from brown to light yellow. On resuming a normal diet diarrhea developed in three out of the four cases.

TABLE I.

Nitrogen Contained in the Foodstuffs Consumed During the Experiment.

	per cent		*per cent*
Dried yeast	9.53	Banana	0.31
Onion yellow	0.31	Sweet chocolate	0.071
Sauerkraut	0.277	Tea biscuit	1.41
Butter	0.055	Bread (baker's)	1.27
Apple	0.096	Tea, 3 gm. to 800 cc.	0.008
Tomato soup	0.68	Tomato	0.71
Rice	1.21	Orange	0.282
Yeast vitamine, 50 cc. autolyzed	0.078		

Case I.—A normal vigorous man, 38 years old, weighing 78 kg. at the beginning of the experiment.

Diet (Gm.).

Yeast period.

Days.	1	2	3	4	5	6	7	8	9	10	11	12
Yeast	30	30	50	60	60	60	60	60	60	60	60	60
Apple	482	482	480	1,045	514	378	250	247	402	277	293	280
Banana	101	127	123	84	74	167	145	91			182	114
Chocolate	21	63	63	63	126	63	130	63	94		94	336
Tea, cc.	300	300	600	300	300	300	900	600	300			600
Butter							55	49			25	
Onion			71	127	192	161	98	68	150			118
Orange							110			131	124	156
Sauerkraut								100				
Tomato								65				
Sugar	14	7	28	14	7							

Rice and bread period.

Date.	1	2	3	4	5	6	7	8	9	10	11	12
Bread			238	163	237	237	238	238	238	....	....	...
Tea biscuit	204	183									....	...
Butter	100	50	62	87	52	74	115	98	114	....	....	...
Rice	200	200	200	200	200	200	200	200	200	....	....	...
Water, cc	3,000	1,776	604	1,031	750	891	779	851	694	....	....	...

Metabolism Experiment. Case I.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
				Normal diet.					
1916	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*	*kg.*		
June									
3		11.38	10.10	1.28	1,700	33.5	79.4		
4		10.35	9.05	1.30	1,740	45.0	78.9		
5		10.35	9.05	1.31					
				Yeast period.					
6	3.60	8.06	6.70	1.36	820	26.0	78.0	−4.46	
7	3.71	11.58	10.95	0.63	1,840	21.0	77.4	−7.24	
8	5.92	11.24	10.61	0.63	1,540		77.4	−5.32	
9	7.97	9.86	7.06	2.80	1,180	52.0	77.1	−1.89	Blood taken.
10	7.21	8.51	6.79	1.72	1,120	33.7	77.1	−1.31	
11	7.13	8.02	6.92	1.10	2,160	30.0	76.6	−0.89	
12	7.34	7.94	6.84	1.11	1,350		76.6	−0.60	Blood taken.
13	7.89	9.81	8.05	1.76	1,270	25.0	76.6	−1.92	
14	6.66	6.10	5.53	0.57	500	20.0	76.4	+0.56	Blood taken.
15	6.47	9.74	9.16	0.58	1,450		75.7	−3.26	
16	6.99	13.62	9.88	3.74	1,760	66.0	75.2	−6.63	Blood taken.
17	7.34	9.76	8.19	1.57	2,120	24.7	75.0	−2.42	
				Rice and bread period.					
18	5.35	5.96	4.39	1.57	380	24.7	76.6	−0.61	
19	5.03	9.40	7.22	2.18	730		77.1	−4.37	
20	5.47	10.12	7.94	2.18	1.450		76.2	−4.65	
21	4.54	8.19	7.42	0.77	1.100		74.6	−3.64	Blood taken.
22	5.45	7.54	6.77	0.77	780	58.0	75.0	−2.00	
23	5.47	6.52	5.75	0.77	460		74.8	−1.05	
24	5.50	8.04	7.27	0.77	660		74.5	−2.54	
25	5.49	5.64	4.87	0.77	340		74.8	−0.15	
26	5.50	6.98	6.21	0.77	400		75.9	−1.48	Blood taken.

Urine Analysis, Case I.

Date	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*
9	0.510	10.6	1.04	0.895	1,120	
12	0.504	10.6	1.57	0.90	1,350	
14	0.304	9.95	0.826	0.53	500	
16	0.790	15.4	0.890	1.32	1,760	
20					1,450	2.10
21	0.623	7.55	1.47	0.469	1,100	1.62
23					460	1.52
24					660	1.78
26	0.024	10.3	1.28	0.388	400	

Blood Analysis, Case I.

Date.	Total N.	Non-protein.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO_2	Volume.	Remarks.
June	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*		*cc.*	
9	3.26	25.4	23.8	0.75	4.0	0.108	46	55	Yeast diet.
12	3.30	29.4	28.5	0.57	2.0	0.106	47	65	
14	5.04	32.7	34.6	0.72	4.2		46	55	
16	3.22	25.1	27.7	0.85	5.0	0.092	45	54	
21	2.87	25.8		1.3	2.8	0.090	41	48	Rice diet.
26	2.57	28.5	32.8	2.2	3.5	0.091	49	41	
Total loss of nitrogen by bleeding.	10.78								
July									
7	2.84	37.8	29.4	0.5	1.3	0.0744			Normal diet.

In considering the results of the metabolism experiment in Case I we find that yeast was fairly well utilized in the middle of the first period, but later was found, to some extent, undigested in the feces. However, in the period in which white rice and bread were given as a control for a vitamine-free diet, the man under experiment went more and more into nitrogen balance without the addition of vitamine. This might be due to the fact that less water was consumed in the latter part of the experiment. A high uric acid content occurred in the blood in the yeast period, and a fairly high sugar content in the urine on the vitamine-free diet.

Case II.—A normal man, 22 years old, weighing 68.4 kg. at the beginning of the experiment.

Diet (Gm.).

Yeast period.

Days	1	2	3	4	5	6	7	8	9	10	11	12	13
Yeast	25	25	35	50	50	50	55	55	55	55	55	55	55
Apple	145.5	434	211	173.5	221	358				207		98	73
Banana	106.1	155	258.5	118			91.5	87.5				117	
Chocolate		21	84	52.5	30.5	94.5		126	84	73.5	115.5	63	
Tea, cc.	300	750	450	450		600		300					
Butter			50			10							
Onion			102				127						
Orange							152				96	79	142
Sauerkraut								150	100	100	100		
Tomato soup		54		50	40	100		62	62	59	62	62	
Sugar	40	24	32	32	24	32	32	24					
Bread										4			75

Rice and bread period.

	1	2	3	4	5	6	7	8	9	10	11	12	13
Bread	228	228	228	228	228	228	228	228					
Butter	100	100	100	100	100	100	100	100					
Rice	175	175	175	175	175	175	175	175					
Water, cc.	700	700	700	700	700	700	700	700					
Yeast, vitamine, as colloidal silica preparation					4.8	4.8	4.1	3.0					

Metabolism Experiment, Case II.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
				Normal diet.					
June	gm.	gm.	gm.	gm.	cc.	gm.	kg.		
3		16.29	15.96	0.33	1,125	17.9	69.1		
4		16.27	15.94	0.33	1,260		69.1		
5		14.19	13.86	0.33	1,140		68.9		
				Yeast period.					
6	4.46	11.25	9.82	1.43	900	23.3	68.4	−7.28	
7	3.97	9.02	7.03	1.99	640	33.0	68.2	−3.96	
8	5.09	8.78	6.68	2.1	950	37.5	67.9	−3.69	
9	5.77	8.19	6.87	1.32	720	38.0	67.0	−2.42	Blood taken.
10	5.90	9.33	8.01	1.32	840		67.0	−3.43	
11	5.90	8.46	7.04	1.42	800	36.0	66.4	−2.56	
12	6.16	7.63	6.20	1.43	1,210		65.7	−1.47	Blood taken.
13	6.25	7.61	6.38	1.23	540	40.0	65.2	−1.36	
14	6.04	8.04	6.80	1.24	545		65.2	−2.00	Blood taken.
15	6.24	6.73	5.65	1.08	600	56.0	65.3	−0.49	
16	6.10	5.99	4.91	1.08	575		65.3	+0.11	Blood taken.
17	6.40	7.57	6.59	0.98	1,020	51.5	65.8	−1.17	
18	6.77	6.57	5.58	0.99	1,250		65.3	+0.20	
				Rice and bread period.					
19	5.07	7.68	6.80	0.88	800	Feces for 4 days	65.0	−2.61	
20	5.07	6.91	6.03	0.88	560		65.0	−1.84	
21	5.07	6.69	5.81	0.88	525		64.6	−1.62	Blood taken.
22	5.07	7.68	6.80	0.88	640	48.5	65.3	−2.61	
				Vitamine addition.					
23	5.15	6.56	5.69	0.87	480	51.4	64.6	−1.41	
24	5.15	6.49	5.62	0.87	680		64.6	−1.34	
25	5.15	7.46	6.59	0.87	750		65.2	−2.31	
26	5.15	6.18	5.31	0.87	490		65.0	−1.03	

Urine Analysis, Case II.

Date.	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar.
June	gm.	gm.	gm.	gm.	cc.	gm.
9	0.354	10.10	1.50	0.604	720	
12	0.348	9.85	1.60	0.930	1,210	
14	0.402	12.10	1.08	0.410	545	
16	0.390	6.32	0.725	0.569	575	
21	0.370	7.54	0.894	0.620	525	1.89
24					680	1.56
25					750	1.78
26	0.417	7.70	1.13	0.475	490	

Blood Analysis, Case II.

Date.	Total N.	Non-protein.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO₂	Volume.	Remarks.
June	gm.	mg.	mg.	mg.	mg.	gm.		cc.	
9	3.26		26.3		3.5			15	Yeast diet.
12	2.78	29.8	26.3	0.24	4.0	0.111	45	83	
14	5.66	30.2	22.1	0.86	8.0	0.100	46	50	
16	3.20	23.7	21.0	1.20	4.8	0.099	46	56	
21	2.65	26.5		0.51	2.0	0.100	41	71	Rice diet.
26	2.72	27.0	29.4		1.5	0.097	43	32	
Total loss of nitrogen by bleeding.............	10.17								
July									
7	2.46	30.0	24.6	0.7	1.7	0.110	48		Normal diet.

In Case II the noticeable factors are the fairly good nitrogen balance obtained, and the high figures for uric acid in blood with yeast. Vitamine gave no definite results in the rice period.

Case III.—A normal man, 20 years old, weighing 65.7 kg. at the beginning of the experiment.

Diet (*Gm.*).

Days.	1	2	3	4	5	6	7	8	9	10	11
					Yeast period.						
Yeast	35	35	45	50	50	55	55	65	55	60	60
Apple	243	138	111	110	86	326	79	404	310	121	
Banana	83	128	138	193	90			81	100	77	78
Chocolate	31.5	63	126	126	273	168	318	147	210	231	136.5
Tea, cc	600	900	900	900	600	1,200	1,400	900	900	900	900
Butter	173	162	99	50	10	60	50				
Onion			70	76							
Orange						152					
Tomato soup				50		100	45				
Tomato										59	63
Bread	50	50			50		50				
Sugar	28	14	49	49	28	80	66	52	49	42	63
					Rice and bread period.						
Bread	200		200	200	210	200	200	200	200	200	
Tea biscuit		185									
Butter	100	100	105	129	110	120	120	120	120	120	
Sugar					25		25	25	25	25	
Water, cc	1,163	1,190	761	1,193	1,308	1,211	954	1,011	1 055	612	
Vitamine						3.75	4.81	4.8	4.16	3.0	

Rice 155 gm. throughout each day of the whole period.

Metabolism Experiment, Case III.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
Normal diet.									
June	gm.	gm.	gm.	gm.	cc.	gm.	kg.		
3		15.38	14.43	1.90	1,000	29.6	65.0		
4		13.95	13.0	1.90	1,020		65.0		
5		13.83	13.37	0.46	1,620		65.0		
Yeast period.									
6	3.63	10.96	10.49	0.47	700	12.8	65.7	−7.33	
7	4.64	11.46	10.47	0.99	600	19.1	65.4	−6.82	
8	4.62	10.18	9.27	0.91	1,140	17.3	64.8	−5.56	
9	6.21	9.97	8.15	1.82	1,000	30.9	64.4	−3.76	Blood taken.
10	6.02	11.88	8.38	3.50	670	34.0	63.8	−5.86	
11	7.00	9.20	7.32	1.88	1,040	62.49	64.1	−2.20	
12	6.63	10.22	8.33	1.89	1,525		63.5	−3.59	Blood taken.
13	6.62	7.53	6.04	1.49	600	45.7	63.7	−0.91	
14	6.07	8.72	7.23	1.49	620		63.2	−2.65	
15	6.72	10.45	7.56	2.89	780	50.2	63.9	−3.73	
16	7.00	13.54	7.48	6.06	650	101.8	63.1	−6.54	Blood taken.
Rice and bread period.									
17	4.47	8.06	7.18	0.88	550	44.9	63.9	−3.59	
18	4.54	8.79	7.91	0.88	780		63.7	−4.25	
19	4.47	8.30	7.42	0.88	780		63.2	−3.83	
20	4.48	7.82	7.56	0.26	720		63.0	−3.34	
21	4.60	7.28	7.02	0.26	500		62.9	−2.68	Blood taken.
Vitamine addition.									
22	4.56	5.39	5.13	0.26	580	26.2 including June 20 and 21.	63.2	−0.83	
23	4.56	6.06	5.80	0.26	520		62.8	−1.50	
24	4.56	7.01	6.75	0.26	510		63.1	−2.45	
25	4.56	7.94	7.68	0.26	1,020		63.2	−3.38	
26	4.56	6.87	6.61	0.26	1,040		63.0	−2.31	

Urine Analysis, Case III.

Date.	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar.
June	*gm.*	*gm.*	*gm.*	*gm.*	*cc.*	*gm.*
9	0.500	11.6	1.6	0.875	1,000	
12	0.412	22.7	1.64	1.18	1,525	
16	0.396	10.5	0.908	0.487	650	
21	0.452	7.45	0.815	0.405	500	0.41
26	0.420	10.1	1.65	0.641	1,040	

Blood Analysis, Case III.

Date.	Total N.	Non-protein N.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO_2.	Volume.	Remarks.
June	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*		*cc.*	
9	3.02	31.2	25.6	0.59	1.8	0.107	49	55	Yeast diet.
12	3.18	28.8	25.7	0.25	1.9	0.104	49	64	
16	3.08	31.8	27.2	0.13	4.9	0.084	47	Lost.	
21	2.66	28.0		0.59		0.088	46	61	Rice diet.
26	2.87	29.0	27.2	2.1	3.0	0.086	45	56	
Total loss in nitrogen by bleeding..............	8.4								
July									
7	2.58	33.0	39.6	0.6	2.8	0.119	46		Normal diet.

Case IV.—A normal man, 32 years old, weighing 63.5 kg. at the beginning of the experiment.

Diet (Gm.).

Yeast period.

Days.	1	2	3	4	5	6	7	8	9	10	11
Yeast......	20	20	40	50	50	55	55	55	55	60	50
Apple......	275	350	328	187	152	323	390		180	95	105
Banana....	202	205	132	121	173	74	73	93			
Chocolate..	84	157.5	84	147	168	147	92	107	126	168	148
Tea, cc.....	300	300	900	1,200	1,500	1,350	1,200	1,800	1,500	950	1,100
Butter.....			30	41			25				
Onion......			10	25			14				
Orange.....					110	100	95		350	70	50
Sauerkraut.								124	250	100	200
Sugar......	14	11	28	60	70	66	58	72	56	56	63
Water, cc..										150	150

Rice and bread period.

Days.	1	2	3	4	5	6	7	8	9	10	11
Bread......	200		200	200	200	200	200	200	200	200	
Tea biscuit......		185									
Butter.....	100	100	100	100	110	115	110	110	110	110	
Water, cc..	1,250	1,155	639	680	891	1,066	821	916	854	673	
Vitamine as Lloyd's reagent...						3.75	4.8	4.8	4.1	3.0	

Rice 155 gm. throughout the period.

Metabolism Experiment, Case IV.

Date.	N intake.	N output.	N in urine.	N in feces.	Urine.	Feces.	Weight.	Balance.	Remarks.
				Normal diet.					
June	gm.	gm.	gm.	gm.	cc.	gm.	kg.		
3		13.07	11.26	1.81	915	31.05	64.1		
4		12.75	11.2	1.55	760	25.3	64.1		
5		13.81	11.0	2.81	745	45.25	64.1		
				Yeast period.					
6	2.91	11.02	9.85	1.17	1,440	18.8	63.5	−8.11	
7	3.03	8.03	6.4	1.63	700	24.4	62.7	−5.00	
8	4.70	10.67	8.23	2.44	840	36.1	62.3	−5.97	
9	5.62	9.54	7.88	1.66	870	28.5	61.8	−3.94	Blood taken.
10	5.97	12.83	8.22	4.61	1,180	72.65	61.9	−6.88	
11	6.28	10.36	8.23	2.13	1,160	62.75	61.5	−4.08	
12	6.31	8.99	6.85	2.14	825		61.2	−2.68	Blood taken.
13	6.00	8.93	7.21	1.72	740	50.9	61.3	−2.93	
14	6.41	9.55	7.83	1.72	660		61.3	−3.13	Blood taken.
15	6.48	10.74	8.76	1.98	770	58.85	61.3	−4.26	
16	5.75	9.55	7.57	1.98	630		61.0	−3.80	Blood taken.
				Rice and bread period.					
17	4.47	6.90	6.09	0.81	420	56.0	61.2	−2.43	
18	4.54	7.08	6.27	0.81	480		61.8	−2.54	
19	4.47	6.44	5.63	0.81	440		61.5	−1.97	
20	4.47	6.18	5.37	0.81	530		61.3	−1.71	
21	4.48	5.74	4.93	0.81	460		60.9	−1.26	Blood taken.
				Vitamine addition.					
22	4.56	5.08	5.08	0.72	410	54.0	61.1	−1.24	
23	4.56	5.58	4.86	0.72	430		61.1	−1.02	
24	4.56	6.23	5.51	0.72	405		61.2	−1.67	
25	4.56	6.34	5.62	0.72	490		61.1	−1.78	
26	4.56	6.63	5.91	0.72	480		60.7	−2.07	Blood taken.

Urine Analysis, Case IV.

Date.	Ammonia.	Urea.	Creatinine.	Uric acid.	Volume.	Sugar.
June	gm.	gm.	gm.	gm.	cc.	gm.
9	0.354	12.2	1.20	0.90	870	
12	0.335	10.6	1.25	0.75	825	
14	0.370	12.8	1.29	0.533	660	
16	0.432	11.6	0.645	0.497	630	
21	0.410	7.14	1.29	0.409	460	0.27
24					405	0.54
26	0.452	9.2	1.20	0.465	480	

Blood Analysis, Case IV.

Date.	Total N.	Non-protein N.	Urea N.	Creatinine.	Uric acid.	Sugar.	CO_2.	Volume.	Remarks.
June	gm.	mg.	mg.	mg.	mg.	gm.		cc.	
9	4.30	34.0	33.6	1.1	4.8	0.119	39	60	Yeast diet.
12	3.90	29.4	31.2					9	
14	3.16	27.2	43.0	0.62	5.9		47	60	
16	3.18	30.2	34.8	1.1	5.6	0.093	49	60	
21	2.92	32.2	29.0	2.2		0.091	43	58	Rice diet.
26	2.59	30.6	33.6	1.1	2.9	0.105	46	67	
Total nitrogen lost by bleeding.	10.16								
July									
7	2.79	32.3	40.3	1.3	1.9	0.12	48		Normal diet.

Besides the blood analysis the blood pressure was taken June 13, and found to be as follows: Case I, 125 mm.; Case II, 130 mm.; Case III, 115 mm.; and Case IV, 120 mm.

Besides the blood pressure the hemoglobin content and also a differential leukocyte count were registered on June 16 on the yeast diet, which figures were found normal:

Case.	Hemoglobin.	Polymorphonuclears.	Leukocytes.	Large mononuclears.	Transitionals.	Eosinophils.	Basophils.
I	88	42	50	1	1	5	1
II	89	69	29	1	1	0	0
III	80	65	32	0	1	1	0
IV	82	66	33	0	0	1	0

As a result of our nutrition experiments with yeast it is shown that the value of yeast as a protein source is not very great. Thomas (18), by comparing the protein substitution value of different foodstuffs, concluded that the biological value of yeast is 70.52 per cent. From our experiments this is certainly not the case, as a large quantity of unutilized yeast passes in the feces and is lost. Yeast, on account of its high purine content, causes a distinct rise of uric acid in the blood, and for this reason cannot be used to the exclusion of all other food. Our experiments with vitamine-free food, in the form of white rice and white bread, confirm the results of Abderhalden, Fodor, and Röse, who found that to obtain a positive nitrogen balance, more white bread is required than whole bread or potatoes. In their experiments as well as in ours, this negative balance occurred in spite of the low nitrogen figures in the feces, and indicated good resorption. Whether this low value of vitamine-free food is due solely to the absence of vitamines, we were not able to prove as our experiments were of such comparatively short duration.

RÉSUMÉ.

Yeast cannot very well be recommended as a sole protein source, as a large part of the yeast nitrogen apparently has no food value. It is badly assimilated and occasions a rise of uric acid figures in the blood. The amount of nitrogen which would be fully adequate in the form of potatoes was proved to be insufficient with yeast. This also applies to our experiment with white bread and white rice. We were unable in our experiment to get a positive nitrogen balance by the addition of vitamine.

In this field of nutrition research our studies have by no means enabled us to pronounce a verdict that yeast possesses no value in dietetics.

Problems of personal idiosyncrasy as to the taste of foods, methods of administration suitable for the particular individual, questions of amino-acid synthesis after the yeast protein and purine bases have entered the organism, are all complex factors which must be taken into consideration.

Just how far anaerobic yeast, when consumed in conjunction with other foods, can be made of any supplementary metabolism value to a large community suffering from dietetic deficiencies of one type or another, offers an inviting field for further research in the domain of practical experimentation.

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THE NUTRITIVE PROPERTIES OF CORN.

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It has long been a matter of common knowledge that an exclusive diet of maize is inadequate for growing animals, but the precise nature of the deficiency is not yet determined. Thus one school of investigators has ascribed the nutritive deficiencies of corn to its lack of certain inorganic constituents. Forbes (1914) states:

> "Our results show that, whatever the protein deficiencies of corn, its mineral deficiencies are more pronounced, since, in balance experiments, the deficiencies in calcium and other minerals are immediately made manifest by negative balances or deficient storage, while the protein deficiencies, whatever their nature, allow liberal nitrogen retention.

The extreme importance of the mineral constituents cannot be denied, but some investigators have laid more stress on the protein deficiencies of corn than does Forbes. For example, Osborne (1913) states:

> "The results when presented leave no doubt that the deficiency observed in the practical feeding of corn meal is explained largely, if not wholly, by the unique chemical constitution of zein which forms such a large part of its protein."

Another suggestion frequently offered is that the growth accessory substances, the so called "vitamines," are present in such small quantities that normal growth cannot occur unless they are supplied in some supplementary food. As yet, however, there is no convincing evidence at hand to support this theory.

EXPERIMENTAL PROCEDURE.

Most of the data described in this preliminary report were obtained by using albino rats as experimental animals. This procedure has proved so successful at various institutions that

THE JOURNAL OF BIOLOGICAL CHEMISTRY, XXVII, NO. 1

further comment on this point is unnecessary. Our choice, however, is explained by the anticipated use of lysine, tryptophane, and isolated proteins. The cost of these would be prohibitive if prepared in sufficient quantity for any of the ordinary domestic animals. During feeding trials the animals were confined in cages of hardware cloth, following closely the procedure of Osborne and Mendel (1911). Distilled water *ad libitum* was given in all cases.

I. Adequacy of the Inorganic Constituents.

This particular problem was only incidental in the original plan, so our data on this point are relatively meager. Some of the animals were given corn alone, others corn plus a salt mixture. A third lot received corn plus an "ash-free" protein,[1] and a fourth, corn plus "ash-free" protein plus a salt mixture. The results are given in Table I.

The results at least have a positive trend. If corn is the sole article of diet the animals decline rapidly in weight and soon die. The addition of a salt mixture,[2] however, permitted growth to occur in a surprising degree. When an "ash-free" protein is added to the corn, in most cases death ensues promptly. The dried blood is a striking exception. At the end of 27 weeks the animals were apparently growing slowly. Possibly they would

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[1] The author prepared these materials originally for another research conducted jointly by the executive, chemistry, and animal husbandry departments of the college. The methods of preparation are described later in this article.

[2] Two salt mixtures were used, apparently with equal success. No. 1 was adapted by Dean Willard, of this college, from a mixture published by Osborne and Mendel. No. 2 approximates a mixture published by McCollum.

	No. 1 *gm.*	No. 2 *gm.*
$CaCO_3$	92	
Ca lactate	8	468.0
$Ca_3(PO_4)_2$	10	
K_2HPO_4	37	280.8
NaCl	20	123.1
Na citrate	15	31.2
Fe citrate	2	23.8
$MgSO_4$		31.72

TABLE I.

Growth (Gm.) of Rats on Rations of Low and High Mineral Content.

	Corn alone.			Corn + salt mixture.			Corn + ash-free egg white.		Corn + ash-free dried blood.		Corn + corn gluten.		Corn + ash-free casein.		Corn + ash-free egg white + salt mixture.			Corn + dried blood + salt mixture.		Corn + corn gluten + salt mixture.		Corn + ash-free casin + salt mixture.	
No. and sex.	♀ 59	♀ 58	♂ 60	♀ 60	♂ 33	♀ 34	♀ 61	♂ 62	♀ 37	♂ 38	♂ 63	♀ 64	♀ 65	♂ 66	♂ 67	♀ 29	♀ 30	♀ 35	♂ 36	♀ 68	♀ 69	♂ 70	♀ 71
Initial weight.	57	57	55	89	48	45	50	52	53	57	47	42	53	89	56	70	62	48	57	45	35	47	50
wks.																							
3	59	58	60	95	65	64	64	78	95	87	55	47	60	79	85	92	97	60	74	78	65	83	80
6	65	61	62	120	100	100	50	72	100	105	72	52	67	84	107	90	115	93	123	115	105	146	105
9	67	58	63	135	135	115	Dead.	Dead.	107	112	74	Dead.	53	77	132	105	122	90	153	140	138	162	135
12	70	Dead.	65	133	160	135			115	117	75		Dead.	Dead.	177	115	132	127	167	167	148	185	155
15	65		63	140	175	135			110	112	62				180	120	130	125	172	165	150	195	165
18	Dead.		Dead.	133	187	140			115	130	Dead.				175	120	140	125	180	175	156	210	172
21				139	200	145			120	130					215	125	145	130	195	180	167	215	175
24				140	220	160			133	140					225	130	140	135	190	200	180	Diet	
27				145	265	190			135	125					225	Diet		145	195	215	205	changed.	
30				125	275	190			Diet							changed.		145	200	225	220		
33				110					changed.									Diet		Diet			
36				120														changed.		changed.			
39				115																			
				Killed.																			

Rats 33 and 34 were mated Mar. 1, and five young were delivered Apr. 4. All were alive at birth, but with one exception they did not survive long. The mother began eating the young almost immediately after delivery, and so in some cases the young may not have met a natural death. Although numerous litters have been reared in this laboratory, this is the only case observed where any of the young have been eaten.

have declined later, but our facilities would not allow us to continue the trial longer.

A somewhat similar experiment has been described by Weiser (1914). His object was to determine the effect of a calcium-poor diet on the composition of growing bones. One group of three pigs received maize and corn gluten, another group of three received the same ration plus about 0.75 per cent of calcium carbonate. Some time after the feeding trial began dried blood was substituted for corn gluten. During the first part of the trial the weights of the lots only were given, as the individual records are not available. During the latter part of the trial the individual weights are recorded. A portion of the record is summarized in Table II.

TABLE II.

Growth (Kg.) of Swine on Rations of Low and High Calcium Content.

	Lot with Ca.	Lot without Ca.	
Jan. 15	17.7		17.6
June 15	41.8		36.8
	No. 1.	No. 4.	No. 6.
July 7	19.6	14.5	9.9
Sept. 24	35.7	12.6	11.1

Even the animals receiving calcium grew slowly, but the increased growth due to its addition to the diet is quite marked.

Data obtained at this institution are not in complete agreement with Weiser's findings. Extensive feeding trials here have been conducted jointly by the executive, animal husbandry, and chemistry departments, using swine as experimental animals. One lot received corn alone, and another corn plus salt mixture No. 1. The figures are very striking, and as they represent the experience of several years, there can be no doubt that they are entirely trustworthy. On corn alone, the animals can barely maintain life. Some of them in fact do succumb, while a few others maintain life in a precarious condition for some months, but gradually gain in weight, and also show a real increase in length and height. After some months the animals grow more rapidly, they take on a more thrifty appearance, and in the course of time reach a long deferred maturity. A striking point in these researches is the failure to derive any benefit from the use of a salt mixture. In contrast to this stands the real value derived from the addition to corn of a protein comparatively free from inorganic constituents. Two substances were used for this purpose; the proteins of blood were used in one trial, and those of egg white in the other. Both of these are comparatively free from calcium and phosphorus, but they were given special treatment to remove from them so far as practicable all inorganic constituents. The blood, obtained from Armour and Co., was defibrinated in their plant, chilled, and shipped here. As soon as possible after its receipt it was diluted to four or five times its volume, acidified with acetic acid, and heated to boiling with live steam. The coagulum was filtered off on a linen cloth, returned to the vessel in which coagulation occurred, and washed with a large volume of boiling water. This was again filtered, and dried at 70-80°C. The egg white used was a dried commercial product. This was dissolved in warm water, and then given practically the same treatment as the blood. The analyses are shown in Table III. All animals drank distilled water during the feeding trial.

TABLE III.

Ash and Nitrogen Analyses of Feeds.

	Total ash.	Ca	P	N
	per cent	*per cent*	*per cent*	*per cent*
Corn	1.57	0.015	0.288	1.57
Blood proteins	1.09	0.0126	0.0824	15.21
Egg white	0.57	0.0110	0.089	14.75
Starch	1.02	0.0132	0.196	0.056

As far as growing swine are concerned, therefore, corn contains sufficient inorganic material for the protein already present. It would seem in this case that the first limiting factor is the protein. It is very certain that corn does not contain enough mineral matter for normal growth. In support of this statement additional data are submitted. Other lots of swine received corn and the "ash-free" protein and salt mixture No. 1. In this case growth was approximately normal.

The final results of these feeding trials are given in Table IV. The upper part of the table gives the composition of the feeds in percentages. Crude fiber is not included here, as the amounts were small, and its value as a source of energy is uncertain. The sum of the protein, ether extract, nitrogen-free extract, and ash is taken as 100 per cent. The lower portion of the table gives the weights of the animals. These weights are the average of the lots. Each lot contained three pigs, except Lots 38 and 39, which contained two pigs each.

TABLE IV.

Growth of Swine on Rations of Low and High Mineral Content.

Lot No.	30	31	32	33	38	39
Ration	Corn.	Corn + salt mixture.	Corn + ash-free blood protein.	Corn + ash-free blood + salt mixture.	Corn + ash-free egg white.	Corn + ash-free egg white + salt mixture.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Protein: Corn	11.20	10.88	10.00	9.55	10.00	9.56
Protein: Supplement			10.51	10.10	10.52	10.01
Fat	4.72	4.58	4.22	4.03	4.22	4.03
N-free extract	82.70	80.30	73.80	70.60	73.80	70.60
Ash	1.55	4.27	1.51	5.76	1.46	5.72
Calcium	0.017	0.66	0.17	0.99	0.17	1.01
Phosphorus	0.33	0.44	0.30	0.51	0.30	0.55
1915	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*
Initial weight: July 3	25.3	26.0	25.0	23.7		
Initial weight: Aug. 2					27.5	28.5
Final weight, Dec. 30	37.6	32.6	82.6	171.6	69.5	192.5

The average weights of the animals give the impression that Lot 32 made a greater gain than Lot 38. The individual records do not altogether support that idea, however, as one pig in Lot 32 made a much greater gain than any other receiving a similar ration.

II. Adequacy of Maize Proteins.

The rate of growth of rats receiving corn plus a salt mixture indicates in some cases an almost surprising efficiency for the corn proteins. This is the more striking if the large percentage of zein in corn proteins is borne in mind. As has often been pointed out, this protein contains neither lysine nor tryptophane. The question is then at once presented, as to whether growth has occurred in spite of the zein, or whether zein in the ration has real value as a protein.

This problem was approached from various viewpoints. In one case the ration of corn and salt mixture was supplemented by zein itself. If the addition of this protein to maize should exert any beneficial action it would seem clear that the zein already in corn played an essential rôle in the ration of growing animals. On the other hand failure of the addition to exert any such action would not necessarily prove that all of the zein already present was without value. Again the data are not conclusive, but it does seem highly probable that the addition of zein not only had no value, but was really detrimental. Although Rat 19 made a fair degree of growth, yet on the whole the animals receiving the addition compare unfavorably with any of the others receiving a protein supplement, especially when it is noted that three of these animals are males. It seems highly probable that the addition of zein had actually lowered the value of corn.

The addition of gliadin to corn was tried because, according to Osborne and others (1915), it contains at most a very small percentage of lysine; they give 0.92 per cent as the probable value. If the addition of gliadin should cause a quickening of the growth curve, it would seem that the slow growth on corn was not due to lack of lysine in the zein. Osborne and Mendel (1911) have already demonstrated that in other respects gliadin is an adequate protein. Though it seems clear that no beneficial results could be ascribed to the gliadin, it also seems equally clear that it had no detrimental action.

Osborne and Mendel (1914) had previously demonstrated that the addition of lysine and tryptophane to zein converted it into a protein adequate for maintenance and growth; so it seemed possible that the addition of these amino-acids to corn itself might convert the latter into a more efficient diet. Accordingly two rats received corn plus 1 per cent lysine, two more received corn plus 0.5 per cent tryptophane, and two more received corn plus

1 per cent lysine and 0.5 per cent tryptophane. In none of the six cases did the addition have any evident value.[3]

The food mixture was prepared by grinding the entire maize kernel to an impalpable powder, and then intimately mixing with it the amino-acids. This dry mixture was fed to the animals without making any effort to form it into a paste, or to give it greater coherence. This procedure has one serious disadvantage in that rats are wasteful feeders, and it is difficult to obtain the exact weight of the food consumed. It seemed impossible to avoid that difficulty however, without encountering others equally serious. Observation, as well as such records as we could obtain, indicated that the rats making even moderate gains on corn alone ate relatively very large quantities of food.

In every case where corn was supplemented with a protein the amount added was such as to make the nitrogen content 3.2 per cent, or the protein content approximately 20 per cent. Thus the corn proteins were slightly less than one-half the total protein. The data are presented in Table V.

For comparison some results summarized in Table I are also presented in Table V along with other material that does not bear directly on the question of zein insufficiency. Considerable variation in the rate of growth is evidenced even among animals receiving the same food. The first two diets given, dog bread and sunflower seed, and corn and casein, are regarded as suitable for normal growth. For reasons that will be given later however, Rats 70 and 71 are considered slightly below normal in rate of growth.

It is evident that maize alone may be sufficient for maintenance and for growth, at least during a period of 7 months. In fact the rapidity of growth that may be attained on this ration is surprising. Another striking point is the very rapid rate of growth on corn when supplemented by casein.

The only notable feature concerning the feeding of egg white and dried blood is the failure of either to exert any beneficial effect. Our data on dried blood when supplied in other food mixtures are incomplete, but the records of Rats 9, 10, 11, and 12 leave

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[3] These feeding trials are now being conducted under different conditions, and the results indicate that the addition of lysine and tryptophane does increase the efficiency of corn proteins.

Growth (Gm.) of Rats on Corn Alone, and on Corn + Various Protein Supplements.

	Dog bread and sunflower seed.		Corn + casein.						Corn.			Corn + egg white.				Corn + dried blood.		Corn + corn gluten.		Corn + gliadin.				Corn + zein.				Corn + lysine.		Corn + tryptophane.		Corn + lysine and tryptophane.		Protein-free milk, butter, starch, and 9 per cent egg white.		Protein-free milk, butter, starch, and 15 per cent egg white.*	
Sex. No.	♀ 77	♀ 78	♂ 70	♀ 71	♀ 73	♀ 74	♂ 75	♂ 76	♀ 60	♂ 33	♀ 34	♀ 72	♂ 67	♀ 29	♀ 30	♀ 35	♂ 36	♀ 68	♀ 69	♀ 7	♀ 8	♀ 23	♂ 24	♀ 19	♂ 20	♂ 21	♂ 22	♀ 16	♀ 15	♂ 13	♀ 14	♂ 17	♂ 18	♀ 9	♀ 10	♂ 11	♂ 12
wks.	57†	49	47	50	82	60	38	34	90	48	45	86	56	70	62	48	57	45	35	51	52	50	55	48	50	53	52	50	67	50	57	50	51	75	51	61	54
3	80	70	83	80	90	95	66	54	95	71	67	110	85	92	97	60	74	78	65	82	78	70	92	75	63	80	65	80	90	70	77	85	77	100	80	105	85
6	117	96	146	105	135	133	110	98	120	105	107	135	107	90	115	93	123	115	105	113	107	92	132	90	84	102	92	105	107	107	105	110	90	120	110	155	130
9	162	122	162	135	166	148	148	136	135	135	112	142	132	105	122	90	153	140	138	130	110	106	160	115	115	110	100	140	120	120	115	130	110	130	130	200	175
12	200	160	185	155	180	160	185	180	133	168	132	150	177	115	132	127	167	167	148	145	120	120	170	125	120			155	135	110	115						
15	210	165	195	165	186	182	228	222	140	180	135	165	180	120	130	125	172	165	150	153	135			140	122			155	140								
18	225	170	210	172	188	185	262	232	133	195	142	160	175	120	140	125	180	175	156	155	139			150	128			145	140								
21	200	185	215	175	197	190	282	242	139	210	145	172	215	125	145	130	195	180	167	175	140			156	147												
24	200	170							140	231	167	170	225	130	140	135	190	200	180	175	140			165	165												
27	215	190							145	265	190	175	255			145	195	215	205	175	150																
30	245	190							125	275	190	170				145	200	225	220																		
33									110			150																									
36									120			160																									
39									115			165																									

	gm.
* Egg white (air-dry)	92
Protein-free milk	125
Butter	144
Starch	176
Agar	25

† Initial weight.

no doubt of the adequacy of egg white when supplied in a suitable mixture. The corn gluten[4] seemed more efficient than either the egg white or dried blood, but it is possible that this result should be confirmed by a larger number of experiments before the fact can be considered established.

A discussion of other features of the swine feeding experiment conducted at this institution is pertinent at this time. The failure of corn as a sole diet for swine may be due to the wide nutritive ratio of corn, or it may be due to the inadequacy of the proteins that are present in corn. To obtain light on that question casein[5] was added to corn in sufficient quantity to double the digestible nitrogen already present in the grain. Corn starch was then added in sufficient quantity to make the nutritive ratio of the total added material, 1: 8.8, equal to that of the corn. Three pigs received this ration, and other lots of three received corn plus various amounts of casein. The quantity of the supplement added was such that Lot 36 received a ration with a nutritive ratio of 1: 6, while this ratio for Lot 35 was 1: 3. The growth on these rations is summarized in Table VI.

TABLE VI.

A Comparison of the Value of Casein and Corn Proteins in the Ration of Growing Swine.

Lot No.	34	36	35
Ration	Nutritive ratio 1: 8.8. Corn 25.00 parts. Starch 21.40 " Casein 3.00 " Salt mixture 1.24 "	Nutritive ratio 1: 6. Corn 50.00 parts. Casein 2.82 " Salt mixture 1.32 "	Nutritive ratio 1: 3. Corn 50.00 parts. Casein 11.68 " Salt mixture 1.54 "
	per cent	*per cent*	*per cent*
Protein: Corn	5.64	10.29	8.77
Protein: Supplement	4.78	4.21	14.91
Ether extract	3.02	4.97	5.92
N-free extract	82.80	76.15	65.80
Ash	3.73	4.33	4.51
	lbs.	*lbs.*	*lbs*
Initial weight, July 3, 1915	72	64	59
Final weight, Dec. 30, 1915	494	600	602

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[4] Kindly furnished by the Corn Products Co. of Edgewater, N. J.

[5] Commercial buttermilk casein obtained from the Beatrice Creamery Co., Topeka. Kansas.

Reference to Table IV shows that lots receiving corn alone or corn plus a salt mixture make practically no growth. Lot 34, as is shown in Table VI, received a diet which supposedly approximates corn plus a salt mixture, but with half the digestible corn proteins replaced by casein. The procedure followed does not permit an exact comparison, but it is evident from inspection of the data that casein is far more efficient for growth than are the proteins of corn. Thus the quantity of casein substituted for corn proteins was 4.78 per cent of the ration, yet this substitution changed the ration from one barely sufficient to maintain life to a ration that sufficed for almost normal growth.

Lot 36 received a ration containing slightly less casein than did Lot 34, but nearly twice as much of the corn protein. In view of the fact that this lot gained almost 30 per cent more than Lot 34, the additional corn protein received by Lot 36 must be ranked as fairly efficient.

Another result worthy of note is the failure to secure more rapid growth by the addition of still larger amounts of casein. McCollum has indicated (1914) that up to a certain limit of protein intake, the amount retained by swine is a certain per cent of that ingested. It does not seem that his generalization would apply to these animals for the protein intake is not excessively high. The nature of the limiting factor is not clear.

Another result, of minor importance in this connection, is the evident inferiority of the blood proteins. Table IV shows that Lots 33 and 39 received rations quite similar, with the exception that in one case the protein supplement was blood protein, in another, egg white. However, the lot that received egg white made a greater gain in 5 months than the blood protein lot did in 6 months.

As has been previously mentioned, some of the rats were able to grow at a fairly rapid rate on corn and a salt mixture, but the swine seemed to utilize this ration for growth much less efficiently. Though this discrepancy might seem to indicate a fundamental difference in their metabolic processes, it is believed that such a result is to be expected. Because of the great disparity in size the energy metabolism of the rat is vastly greater than that of swine, and it ingests much more food per unit body weight. An excellent illustration of this fact is found in a summary by Armsby (1914) of Pettenkofer and Voit's results. For example, a dog weighing 3.10 kilos produces 2.41 times as much heat per unit body weight as does a dog weighing 30.66 kilos. On comparing rats and swine the difference is found to be much greater. According to Knapp (1908) the energy requirement per day of a 200 gm. rat is approximately 50 calories, or 250 calories per kilo. Comparable data concerning swine are not at hand, but the results obtained in the swine feeding experiment just mentioned will serve for purposes of comparison. The average weight of the pigs in Lot 36 for the last 30 days of the feeding trial was 86.4 kilos. The average daily feed consumption during that time was 1.64 kilos. The analyses of the ration are not complete enough for an exact calculation of its calorific value, but it is approximately 5,745 calories. The energy consumption per kilo of these animals, then, is 66.5 calories, or approximately one-fourth of that of a 200 gm. rat. The experimental procedures do not permit a precise comparison, but in all probability the data submitted here tend to diminish rather than exaggerate the differences. Although the energy requirement per unit body weight is much higher for the rat, the protein requirement is generally believed to be little if any higher. In other words, after each animal has satisfied its calorific needs, the rat has a much larger nitrogenous residuum with which to build new protein tissue.

III. The Presence of Growth Accessories.

A number of investigations, notably those of Osborne and Mendel, McCollum, and Funk, make it highly probable that a normal diet must contain certain "accessory" substances. These are of unknown chemical composition, and the quantity required is undoubtedly small, but nevertheless they are indispensable components of an adequate diet.

The suggestion has frequently been made that the slow rate of growth obtained when corn is the sole article of diet is due to a lack of one or more of these accessory substances. The literature on this particular point is not abundant, and is both confusing and conflicting. It might be well to note at the outset that some investigators, Abderhalden (1913) and Röhmann (1916), question the necessity of assuming that any such food accessories, the so called vitamines, exist. Their attitude, however, is not the one commonly taken.

Most of the experiments described in this paper did not include the vitamine factor, but some of the facts presented will bear inspection from that standpoint. It is evident that, if the existence of these accessories is conceded, corn contains them in some degree. In this laboratory rats were brought to apparently normal maturity on corn and a salt mixture as the sole diet. Schaumann (1915) states that corn is "sufficient" for pigeons and rats, but insufficient for rabbits, goats, and guinea pigs. Funk (1913) has shown that the outer layer of corn contains an antineuritic substance, and a recent article by Willard (1916) states that this fact was also discovered independently by J. S. Hughes of this college. Hughes fed polished rice to pigeons until they had developed extreme cases of polyneuritis. They could scarcely move, and would make no effort to swallow. In many cases death was unquestionably only a matter of hours. If a few grains of corn were forced into the crop at this stage, the birds would make a swift recovery, and in 1 or 2 days present a wholly normal appearance. It is evident therefore that some of these accessories are present in corn.

Voegtlin (1915) in an article on the effects of a vegetable diet says comparatively little concerning accessory substances, but gives the impression that satisfactory growth could not be attained by animals fed on corn alone. Slonaker (1912) also states that a strictly vegetable diet is unsatisfactory for growing rats.

The original data submitted in this paper make it evident that rats may slowly grow to apparently normal maturity on corn alone, though some do not succeed in doing so. When corn is supplemented with casein growth occurs at a fairly rapid rate. The blood preparation seems slightly less efficient, and the egg white considerably less. Inasmuch as casein ordinarily carries large quantities of the accessories, the ineffectiveness of the egg white might seem due to a lack of those substances, and that explanation readily suggests itself. That the difference is not due to poor quality of the protein is shown by the behavior of Rats 11 and 12, Table V. These animals received egg white along with starch, butter, and protein-free milk, and grew at the maximum rate. Additional support for the views that egg white may be lacking in accessories is found in McCollum's (1915) experience in using egg white as an adjunct to polished rice. The failure of rats to gain on polished rice has been ascribed to protein insufficiency, so McCollum supplemented the rice with ash-free egg white and a suitable salt mixture. The rats failed to grow normally on such a ration, however, thus indicating a lack of growth accessories in the egg white.

The "ash-free" casein, viewed from that standpoint, may have exerted unexpected efficiency. The only object in preparing it, however, was to obtain a product relatively free from calcium, so the material was not extracted with alcohol or ether, and no effort was made to free it of accessory substances. It is also well to note that the animals receiving such material did not grow as rapidly as those receiving the crude product. In Table V, Rats 70 and 71 received the ash-free casein, and these animals grew at a distinctly slower rate than those receiving crude casein. Corn gluten exhibits surprising value when used in this connection. As our preparation contained 4.4 per cent of ether extract, some interest is attached to Funk's (1914) statement that corn products containing considerable quantities of corn oil are richer in these unknown substances. In view of the relative superiority of casein as a supplement to corn, there is a temptation to give great weight to the vitamine factor and some effort is being made in this laboratory to investigate the theory in an experimental way. It is generally believed that an adequate diet becomes "insufficient," when heated to high temperatures, and a voluminous literature has accumulated on using such material in feeding trials. The usefulness of the procedure is open to question, as it is conceivable that factors other than "vitamines" might be subject to alteration. The method was followed here in a small way, however, for if the accessories are destroyed by heat, and rats are able to grow on heated food mixtures consisting chiefly of corn, then failure of the animals to grow on maize could not be ascribed to lack of accessories in the grain. If, on the other hand, rats fail to utilize heated foods for growth, the interpretation is not so obvious. Accordingly enough egg white to make a total of 20 per cent protein was added to corn, along with 4 per cent salt mixture, and the material was then heated for 6 hours in an autoclave at 30 pounds' pressure. Four animals were given this ration, and their behavior on such a diet is recorded in Table VII.

TABLE VII.

Growth of Rats on a Ration Subjected to High Temperatures.

Rat No.	79	80	81	82
Initial weight.	40	43	50	42
wks.				
3	55	53	62	Dead.
6	58	57	61	
9	72	68	76	
12	70	65	87	
15	75	85	90	
18	68	87	87	
21	Dead.	88	91	
24		64	66	
27		Dead.	Dead.	

None made any noteworthy growth, and all died comparatively soon, without any evident cause, excepting that the food had been heated.

SUMMARY.

The evidence indicates that when corn is fed to rats as the sole dietary the mineral constituents are the first limiting factor, and then the protein. In the case of swine these findings are apparently reversed. Protein is here the first limiting factor, and then the mineral element. The data also indicate that the corn proteins are less efficient for growth than casein. The addition of lysine and tryptophane to maize did not increase its efficiency for growth. The addition of some of the adequate proteins (egg white) seemed of only slight benefit. In view of these facts it seems possible that one of the limiting factors in corn as a food for growing rats is one or more of the growth accessories.

Young rats on a corn diet grow more rapidly when the grain is supplemented with casein than when supplemented with egg white. This observation assumes added significance since food mixtures containing protein-free milk, butter, and egg white are more efficient for growth than mixtures of corn and egg white, even though the protein of the corn mixture furnishes a relatively higher per cent of calories than does the protein of the more efficient diet.

Autoclaved corn mixtures failed to maintain body weight, and ultimate failure resulted.

Since swine grow rapidly on a mixture of corn and egg white, it is believed that corn contains sufficient of the growth accessories for normal growth in swine.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF ACETONE IN URINE.

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Frommer[1] in 1905 used salicylic aldehyde as a qualitative test for the detection of acetone in the urine. Tiemann and Kees[2] had already studied this acetone compound and Fabinyi[3] in 1900 carried the investigations still further. The alkali salt of Fabinyi's dioxydibenzene acetone

$$OH - C_6H_4 - CH = CH - CO - CH = CH - C_6H_4 - OH$$

is the compound, the red color of which was made use of by Frommer in his acetone test. Frommer's qualitative test is as follows:

> "To 10 cc. of urine about 1 gm. KOH is added. Before solution has occurred 10 to 12 drops of a 10 per cent solution of salicylic aldehyde in absolute alcohol are added and the mixture warmed to about 70°C. At the zone of contact of the alkali and urine an intense purplish red ring develops in the presence of acetone."

Frommer claimed for his test the advantage over others in that it was specific for acetone as the presence of diacetic acid did not give the color reaction. Bohrisch,[4] made a critical study of the various qualitative tests and confirmed Frommer's claim of superiority in his test, and advised its general use. The specificity of the reaction suggested its use in the development of a colorimetric method for the quantitative estimation of acetone, using the distillate of the urine (which naturally includes the preformed acetone and that derived from the decomposition of diacetic acid), realizing the advantages such a method would possess in rapidity, specificity of color, and simplicity. The Messinger[5] iodimetric method is the one generally in use. Marriott[6] recently described a nephelometric method for the estimation of minute quantities of acetone in blood and tissues, which method in general was applied by Folin and Denis[7] to urine. The aim of the writer was to make the colorimetric method as simple and rapid as possible without sacrificing accuracy, making it adaptable also to clinical work. It is not a micro method, though applicable to minute quantities when a concentrated acetone solution obtained by a second slow distillation is used. All the above mentioned methods, excepting that of Folin and Denis, require a second time-consuming distillation to make it certain that acetone *only* is included in the result obtained.

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[1] Frommer, V., *Berl. klin. Woch.*, 1905, xlii, 1008.

[2] Tiemann, F., and Kees, A., *Ber. chem. Ges.*, 1885, xviii, 1964.

[3] Fabinyi, R., *Chem. Zentr.*, 1900, ii, 302.

[4] Bohrisch, P., *Pharm. Zentralhalle*, 1907, xlviii, 207.

The determination of β-oxybutyric acid by the Shaffer[8] method, in which the acid is oxidized by bichromate, is in reality an acetone determination; he used the Messinger method for determining the acetone. If glucose is present in the urine it must first be removed, since there goes into the distillate, in addition to the acetone, other volatile substances that absorb the iodine. The colorimetric method is directly applicable to the determination of acetone in the distillate; the sugar in the urine does not interfere with the reaction, since the volatile substances derived from the oxidation of the glucose *do not give the color* reaction.[9] The removal of the sugar, which Shaffer accomplished by basic lead acetate, and a second distillation, is rendered unnecessary.

By driving out all the preformed acetone before the distillation by means of an air current (Folin), it is possible to determine indirectly the amount of preformed acetone by subtracting the acetone derived from diacetic acid from the total acetone.

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[5] Messinger, J., *Ber. chem. Ges.*, 1888, xxi, 3366.

[6] Marriott, W. M., *J. Biol. Chem.*, 1913, xvi, 281.

[7] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xviii, 263.

[8] Shaffer, P. A., and Marriott, W. M., *J. Biol. Chem.*, 1913, xvi, 265.

[9] 3 gm. of glucose were oxidized by potassium bichromate under conditions similar to those given in the text for the β-oxybutyric acid determination. The single distillate absorbed 12.15 cc. 0.1 N, and another sample 18.1 cc. 0.1 N iodine solution. 2 cc. of the distillate did not give any color reaction, employing the method advised in the text.

If sugar is not present in the urine the preformed acetone may be determined directly from the urine and by the subtraction of the preformed acetone from the total acetone, we may calculate the amount of diacetic acid. The presence of sugar in the urine used directly disturbs the intensity of the color reaction.

Method.

Three solutions are necessary in the estimation of the acetone: (1) A 10 per cent salicylic aldehyde solution in 95 per cent alcohol (10 gm. salicylic aldehyde made up to 100 cc. by adding 95 per cent alcohol). (2) 100 per cent KOH solution (to 100 gm. KOH, solid, are added 60 cc. distilled water). (3) The standard solution. It was found that the most convenient way to prepare the standard was by checking the concentration of the acetone (c.p.) by the Messinger method and then making a solution such that 2 cc. should contain 0.1 mg. of acetone. On allowing the standard solution to stand for a long period of time, Marriott[6] noticed the effect of polymerization on the standard solution used in his nephelometric method. It is advisable to renew the standard after 10 days, though it was found that there was no appreciable change in the color intensity after the standard had stood 2 weeks, when permanency of the color was controlled by a freshly made standard and also by a bichromate solution.

In performing the test, urine sufficient to contain 8 to 24 mg. of acetone (usually 25 to 100 cc.) are measured by a pipette into a 750 cc. flask; 5 cc. of concentrated H_2SO_4 are added and the volume is made up to about 300 cc. with distilled water. The whole is distilled for 20 minutes, using a Liebig condenser. The receiver should dip under the liquid in the receiving flask to which has previously been added 25 cc. of distilled water. The distillate is transferred to a 200 cc. normal flask.

The residue of urine from which the total acetone was obtained can be used for the determination of β-oxybutyric acid (Shaffer). Dilute the residue to about 400 cc. with distilled water; add 10 cc. concentrated H_2SO_4; distill again for 2 hours, adding drop by drop 200 cc. of a 0.5 per cent solution of potassium bichromate (for urine containing sugar use 1 per cent). The solution should be kept at moderate boiling so that at the end of 2 hours the distillate can be made up to 250 cc. in a normal flask. The acetone is determined in the distillate as described below.

2 cc. of the distillate are measured into each of two large test-tubes, for duplicate determination. 2 cc. of a 100 per cent KOH solution are added, mixed well, and then 1 cc. of 10 per cent salicylic aldehyde is added. Immediately the tubes are placed in a water bath at 45–50°C. for exactly 20 minutes to develop the color. During this time the solutions should be thoroughly mixed several times. 2 cc. of the standard solution, containing 0.1 mg. of acetone, are prepared similarly and synchronously. The tubes are removed after 20 minutes, 10 cc. of distilled water added to each tube, the contents cooled, and transferred to 25 cc. normal flasks filled to the mark with distilled water.[10] The reading should be made in a Duboscq colorimeter within 30 to 45 minutes from the time of the addition of salicylic aldehyde, setting the standard at 15 mm.

As above stated, if sugar is not present in the urine, the preformed acetone may be determined directly. The urine is made alkaline with a few drops of concentrated KOH, filtered, and the filtrate used for the direct determination of the acetone (preformed), as described above.

Since the development of the color and its intensity depend on the concentration of the reagents, special care should be taken with exact measurements. The amount of salicylic aldehyde and of KOH, and the time for the color development were tried out systematically and found to be most satisfactory when the above procedure was followed. By using pure acetone solution and urine with known amounts of acetone added, it was found in several determinations that the limit of error was ±2 per cent. The colorimetric method generally gives a lower result than the iodometric.

SUMMARY.

A colorimetric method for the quantitative determination of acetone in urine is presented, based on the red color of the alkaline salt of dioxydibenzene acetone, which was first used by Frommer as a qualitative test for acetone. The method is also recommended for clinical use on account of its simplicity, accuracy, and rapidity of estimation.

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[10] In case the solution presents too intense a color, instead of using 25 cc., the volume may be made up to 50 cc. and the result multiplied by two.

THE TOXICITY OF CAROTIN.

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In the course of an investigation of the effects of the bleaching of flour by chlorine gas, it became necessary to ascertain the possible toxicity of the pure pigment matter, both bleached and unbleached. As there could be found in the literature no report of any study of the possible physiological action of coloring matters of this class, it has seemed advisable to publish a brief account of our work, although the results are entirely negative.

It is quite certain that the coloring matter in flour is the same as that in carrots—carotin. Wesener and Teller ascribed the color in flour to carotin in 1911,[1] and suggested this in 1909. Monier-Williams[2] has advanced further proof that the coloring matter in flour is carotin, which is called "carrotene" by him.

The amount of carotin in flour is very small. Monier-Williams found in flour in parts per million as follows:

	Unbleached.	Bleached.
Flour A (photographic)	1.4	0.90
" A (colorimetric)	1.3	0.81
" B (")	2.0	1.28

The color is usually obtained from carrots, which contain it in relatively large quantities. Willstätter obtained 125 gm. from 5,000 kg. of carrots. Monier-Williams gives 2 gm. as the yield from 100 lbs. of carrots. 1.3 gm. coloring matter was obtained by us from 54 lbs. of carrots.

We obtained the coloring matter as follows: 54 lbs. of carrots yielded 21.2 kg. cleaned and trimmed material, which was ground in a meat grinder. The ground material was desiccated first by alcohol and then by distillation at 80–90° *in vacuo*. The dried mass was extracted with boiling 95 per cent alcohol. The alcohol extract yielded an extract with chloroform which was saponified with alcoholic potash. After the alcohol was removed by distillation, the residue was dissolved in water and extracted with ether. This ether extract weighed 11.2 gm. and consisted of colorless crystals mixed with carotin. The pigment was concentrated by dissolving the entire mass in carbon bisulfide and alcohol with the subsequent addition of enough water to cause the carbon bisulfide to separate, carrying with it most of the color. Several such treatments gave finally a highly colored mass that left practically nothing in the alcohol-water layer. This was now treated with alcoholic potash to remove with certainty all carbon bisulfide. After removing the alcohol the aqueous solution of the residue yielded an ether extract containing 1.3 gm. of concentrated pigment.

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[1] Wesener, J. A., and Teller, G. L., *J. Ind. and Eng. Chem.*, 1911, iii, 912.

[2] Monier-Williams, G. W., *Reports, Local Gov't Board (Great Britain), Pub. Health and Med. Subjects, M Series, No. 73,* 1912.

One-half of the pigment, 0.65 gm., was chlorinated as follows: The ether solution was shaken with a water solution of chlorine (6 cc. Cl_2 solution containing 0.01443 gm. Cl_2 per cc.). 0.65 gm. carotin, $C_{40}H_{56}$, requires by theory 0.0861 gm. Cl_2 to form $C_{40}H_{56}Cl_2$, and our material used 0.08658 gm. Cl_2. The color of the ether solution decreased and the Cl_2 odor vanished. The chlorinated product weighed about 0.09 gm. more than the initial amount.

In subsequent experiments we obtained but 1.2 gm. pigment from 200 lbs. of carrots, which vary greatly in pigmentation at different seasons.

This purified pigment was used in the following experiments. As it is soluble only in fat solvents, which are all more or less toxic, it was used dissolved in sterile olive oil. This would seem to be a suitable solvent, however, as shown by the efficiency of phlorhizin dissolved in oil when used in metabolism studies. Because of the extremely minute quantity of coloring matter contained in flour, it was not feasible to conduct experiments with carotin from flour, as only 1 gm. of color is contained in 1,000 kg. of flour, and in view of the demonstrated identity of the coloring matter of flour and carotin from carrots the investigation of the latter seemed sufficient.

Two pairs of male guinea pigs were given intraperitoneal injections of carotin dissolved in olive oil and sterilized by brief heating on the water bath.

N 1. Wt. 339 gm., given 3 cc. oil with 0.100 gm. natural carotin.
P 1. Wt. 316 " " 3 " " " 0.100 " chlorinated carotin.
N 2. Wt. 265 " " 5 " " " 0.200 " natural carotin.
P 2. Wt. 245 " " 5 " " " 0.200 " chlorinated carotin.

These animals were kept under close observation for 12 hours, the temperature being taken every 2 hours. At the end of 4 days, during which they were frequently examined, they were killed with illuminating gas. Practically no effects were observed. All showed a rise of 1–2°F., 2 to 6 hours after the injection, but so did control pigs given olive oil alone. They all remained quiet but alert, and ate as well as the control pigs. The urine at no time showed albumin or sugar, but was of a deep reddish brown color. The autopsies showed no changes except the presence of a considerable amount of fatty yellow material in the peritoneum, but much less than the amount injected. There were no signs of inflammation or other local changes. All the viscera seemed normal in both gross and microscopic examination.

Evidently carotin possesses no considerable toxicity, if any, and chlorination does not make it demonstrably toxic. The quantities of carotin used in these experiments are enormous in comparison to the amounts that could be obtained from any food, even carrots. The larger dose used, 0.2 gm., is as much as is contained in 200 kg. of flour, or as lutein in 4,000 cows' ovaries (Escher[3]).

Local or Cellular Toxicity.

Although the absence of either symptoms or anatomic evidence of irritation after intraperitoneal injections of carotin indicated that it can have little or no local effect, this was determined more definitely by a series of intradermic tests. This method, devised and used in this laboratory by Dr. Corper[4] for the study of local toxicity, has been found very useful for this purpose. The principle and method are similar to those used in the intradermic

[3] Escher, H. H., *Z. Physiol. Chem.*, 1913, lxxxiii, 198.
[4] Corper, H. J., *J. Biol. Chem.*, 1915, xx, p. xxi.

injection of tuberculin or diphtheria toxin. As the two preparations to be compared are injected side by side in the same animal, no individual peculiarities of the animal enter as a source of confusion or error.

The carotin, dissolved in varying concentration in olive oil, was injected in parallel sites intradermically in 0.2 cc. quantities into two guinea pigs. The effects were the same in both.

Carotin dissolved in olive oil to the strength of 10 per cent seems to have a very slight local toxicity, as shown by the development of a little edema and swelling. At 1 per cent the results are very little different from those with olive oil alone. These results are not appreciably different with chlorinated and natural carotin. Olive oil containing very small amounts of carotin (0.1 per cent) seems to cause, if anything, slightly less protracted swelling than olive oil alone; possibly a slight irritant effect from carotin hastens absorption.

These experiments indicate that even in relatively very large doses carotin, whether in its natural state or saturated with chlorine, is almost entirely devoid of toxicity. Such large amounts as 20 mg. injected intradermically cause only a local edema and inflammation, but no necrosis.

The extensive studies of Palmer and Eckles[5] indicate that carotin is almost universally distributed throughout all animal bodies, coming chiefly, if not solely, from the food. Our few experiments seem to be sufficient to warrant the assumption that any such quantities as can ever accumulate in the tissues have no harmful effects.

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[5] Palmer, L. S., and Eckles, C. H., *J. Biol. Chem.*, 1914, xvii, 191.

CLINICAL CALORIMETRY.

XVIII. THE NUMBER OF PLACES OF SIGNIFICANT FIGURES IN THE DATA OF METABOLISM EXPERIMENTS.

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In the publications from this laboratory and many others results have often been expressed with more decimal places than are justified by the accuracy of the methods or the significance of the findings. This involves a waste of time and printers' ink and gives the reader a false impression of the work. It is extremely desirable to get along with the smallest number of digits that will do justice to the experiment and yet allow other investigators to recalculate the work.

In the publication of data three factors must be considered: (1) the accuracy of the analytical results; (2) the possible errors in the methods of calculation; and (3) the significance of the findings. It may be well to consider them in this order.

There is great variation in the accuracy of the different methods of analysis used in metabolism experiments. An attempt is usually made to keep all possible errors within the limits of 1 per cent, but few investigators believe that they can do this in all their work. There are some methods in which the error may be distinctly less than 1 per cent but they are usually affected by some other factor in which the error is much greater.

Holman,[1,2] in his standard work on computations, gives, among others, the following principles and rules which are well worth repeating:

“Retain everywhere enough places to correspond to two unreliable places in the final result; the direct object of this is to keep the first place

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[1] Holman, S. W., Computation Rules and Logarithms, New York, 1915.

[2] Holman, Precision of Measurements, New York, 2nd edition, 1904.

of unreliable figures in the final result substantially free from the accumulated rejection errors.

Exceptions: A final result is seldom stated to more than one uncertain place unless the uncertainty of that place is small (say plus or minus fou or less).

In multiplication or division, the percentage accuracy of the produc or quotient cannot exceed that of the factor whose percentage accuracy is least.

In addition or subtraction, the result cannot be accurate beyond the first decimal place which is inaccurate in any component.

In casting off places of figures, increase by 1 the last figure retained when the first left-hand rejected figure is 5 or greater; otherwise leave it unchanged.

A mean or average should always be carried to two unreliable figures.

A mean is more reliable than the single observation from which it is computed (in proportion to $\sqrt{n}$, the square root of the number of observations)." Undue importance should not be attached to this numerical relation when the number of observations is very small, as for instance when not exceeding 5 or 10.

"In direct multiplication or division, retain in every factor, product, and quotient throughout the entire process, and in final results, for an accuracy of about one per cent, or worse, four places of significant figures; one-tenth per cent, or worse, five places of significant figures; and so on.

If the multiplication or division is performed by means of logarithms the mantissa should contain as many places as are required by the foregoing rules for the direct process; *i.e.*, for about one per cent, or worse use four place tables; one-tenth per cent, or worse, use five place tables.

In addition or subtraction . . . . , for an accuracy of about 1 per cent, or worse, carry the result to four places of significant figures, etc.

If a single observation deviates widely from the others of the series and no source of error can be discovered it should be published but not used in determining the mean except as follows.[3] "Take the mean and the (average deviation) *a.d.* from this mean of the observations omitting the doubtful one. Find the deviation (*d.*) of that one from the mean Then reject the observation if *d.* > 4 *a.d.* . . . . Something must also be left to the judgment of the observer as to the propriety of making a rejection."

As Holman[3] goes on to say: "There is a tendency, especially among inexperienced observers, to become biassed by the first one or two readings of a series, and to reject, without recording it, any later one which does not closely accord with these, tacitly assuming it to be faulty. This is an essentially vicious practice which cannot be too carefully avoided Other things being equal the later observations are entitled to greater rather than less weight than the earlier ones, and no result should be rejected without sufficient warrant.

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[3] Holman, Precision of Measurements, New York, 2nd edition, 1904, 30

The rules[4] are so framed that, barring mistakes, the greatest possible computation error entering into the result of any ordinary computation (*e.g.*, one involving a total of not much exceeding twenty component numbers, steps, or operations, where a rejection error may occur) shall not be sensible compared with the errors of the measurements or data, or shall not sensibly affect the accuracy of the results. They are, therefore, *safe* rules in the worst possible cases. But in order to be so they are necessarily more than sufficiently stringent for some classes of comparatively rough work, where the *infrequent* undetected entrance of a computation error two to four times as large as the experimental error would be permissible. For such work, one less place of figures may be used, but when the rules are thus relaxed the possible consequence should be borne in mind, and special scrutiny applied to the various stages of the computation, special attention being directed to quantities beginning with 1 or 2."

The maximum rejection error possible in any given equation can be readily calculated from the formula $\frac{5n}{10^r}$, n representing the number of factors, quotients, and products at which rejections are made and r representing the last place retained. 5 is, of course, the maximum rejection error in any one number and it is conceivable that it might be rejected in the same direction in every case. The greatest percentage error will occur if every factor begins with the digits 10. In order to make $\frac{5n}{10^r} = \frac{1}{100}$ or 1 per cent we can have the following combinations: $r = 3$ and $n = 2$, or $r = 4$ and $n = 20$. Therefore, if we use only two factors we need retain only three places to have the maximum error 1 per cent. If we have 20 factors, quotients, etc., we must retain four places. It is extremely improbable that the figure rejected would be +5 or −5 in every case and the natural tendency would be for the rejections to neutralize each other. It is also improbable that all the factors would begin with 1. If, for instance, any factor began with 5, the above percentage error would be divided by 5.

In some of the calorimeter calculations there are as many as fifteen or twenty multiplications, divisions, etc., and it is obvious that for such work four places should be retained until the end. On the other hand, analyses of food, urine, etc., involve simple calculations with only three or four factors, some of them bein constants. For such cases the average error would be sma and the maximum error infrequent if the fourth place of eac titration or weighing were rejected at the start. If n were the maximum error would be $\frac{5+5+5+5}{10.0 \times 10.0 \times 10.0} = \frac{20}{1000}$. Th probable error would be more like the following exampl $\frac{5-3+4-1}{15.0 \times 84.0 \times 32.0} = \frac{5}{4032}$. Even if the maximum error did o cur it would be diminished in the averages. The safest rul however, is to retain through the calculations four places whe ever possible. There is no need of retaining five places in an metabolism work except, perhaps, in a few of the calorimet calculations where we are dealing with small differences betwee large numbers. The use of five place logarithm tables can b almost entirely abandoned if the interpolations be carefully mad in four place tables.

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[4] Holman, Computation Rules and Logarithms, New York, 1915, p. xliii.

If slide rules be used, it is usually considered that a 20 inc rule corresponds to a four place logarithm table.

When we come to the publication of the results the matte is entirely different. The calculations have been made and th dangers from rejection errors have been passed. Subsequen investigators may recalculate the findings but such recalculation are seldom complicated and do not involve many divisions o multiplications. After all, a man who recalculates the work from another laboratory does not expect the error to be less than per cent.

Even the man who publishes does not often claim greate accuracy than this. Were the analytical results accurate withi 0.1 per cent, there would be no significance in such accuracy It is hard to conceive of a 1 per cent difference causing any chang in the interpretation of any one metabolism experiment. A per cent difference would be of significance only in the mean of a large number of experiments. If final results are expressed in three places they will always be within the 1 per cent limit and except for numbers beginning with 1, they will be far within this limit.

Each investigator will probably decide for himself the numbe of significant figures that he will publish. It is the purpose o the present paper to standardize the number of places used in the publications from the Russell Sage Institute of Pathology. If the number of places is smaller than heretofore used it must not be felt that the accuracy of the work has been impaired or has been considered inferior to that of other laboratories. Analyses of food, urine, and feces are always made in duplicate and standard methods used. The bomb calorimeter is of the best type made. The respiration calorimeter is probably the most accurate machine of its kind ever constructed. In the metabolism ward the collection of specimens is all that could be desired except in the case of very stupid patients. An endeavor is made to keep the administration of foods to the patients as accurate as the analytical methods. Only foods which vary but slightly in composition are used and these are weighed before cooking, samples being taken from time to time for analysis. The most uncertain quantity is the patient himself or rather the physiological law which is being investigated. Nature does not work within 1 per cent limits, especially in disease.

A table is given showing the number of places to which results will be expressed in the publications from this Institute. In most cases three significant figures will be used; in some where the analysis is not quite so exact only two will be given. In some of the analyses of food, blood, and feces, either two or three figures will be used, depending on the amounts present and the methods of analysis. It must not be felt that this table is intended to disparage the accuracy of any particular method of analysis. The rejections of the third and fourth places have been made chiefly because they lack significance. For example, we could determine the total nitrogen of the urine and feces to 0.01 gm. but if we did not analyze the sweat, the first decimal place in the total excreta nitrogen might show a considerable error. Again, the third decimal place in the respiratory quotient is variable and of no significance except in alcohol checks and in severe diabetes where it is of considerable interest and of approximate accuracy in many cases.

SUMMARY AND CONCLUSIONS.

In metabolism work the analytical error is seldom much less than 1 per cent. A variation of 1 per cent in the result of an experiment would not change its significance or affect its interpretation. For these reasons, it seems unnecessary to publish more than three significant figures in the tables of data and in some cases it is not worth while to publish more than two. In order to avoid the accumulated rejection error it is advisable to retain four figures wherever possible in the calculations and reject the fourth digit only in the final result.

TABLE I.

The Number of Places of Significant Figures Adopted as Standards in the Publication of Metabolism Data.

	No. of significant figures.	Examples.		Unit.
Urine.				
Volume	3	1,560.	654.	Cc.
Total nitrogen	3	12.4	7.45	Gm.
Glucose	3	28.2	9.84	
Ammonia N	3	1.24	0.777	
Urea N	3	11.3		
Creatine N	3	0.013		
Creatinine N	3	0.658		
Uric acid N	3	0.451		
Purine base N	2	0.031		
Amino-acid N	3	0.681		
Sodium chloride	3	14.5		
Total sulfur	3	0.902		
Total phosphorus	3	1.46		
Acetone	3	5.23	0.999	
Diacetic acid	3	4.43	0.851	
β-Oxybutyric	3	24.5	2.45	
Acidity 0.1 N	3	246.		Cc.
D : N	3	3.65		
Feces.				
Nitrogen	3	1.34	0.971	Gm.
Fat	3	12.5	6.34	
Carbohydrate	2	1.2		
Ash	2–3	0.98	2.45	
Moisture	3	56.4		

TABLE 1—*Continued.*

	No. of significant figures.	Examples.		Unit.
Food (analyses).				
Heat of combustion	3	3.24		Cal.
N analysis	3	10.1		Gm.
Protein (N × 6.25)*	3	63.1		
Ether extract	3	17.5		
Phosphorus	3	1.52		
Carbohydrate	3	83.4		
Sodium chloride	3	1.33		
Moisture	3	54.5		
Food (given in ward).				
Calories	3	2,340.		Cal.
N	3	14.5		Gm.
Fat	2	24.		
Carbohydrate	2	250.		
Sodium chloride	2	16.		
Water	2	2,500.0		
Blood.				Mg.
Urea N	2–3	102.	23.	
Non-protein N	2–3	201.	52.	
Glucose	2–3	132.	89.	
Creatinine	2	13.	1.3	
Uric acid	2	11.	2.1	
Chlorides	3	592.		
Fat	2	230.	140.	
CO_2 combining capacity	2	69.		Volume per cent.
Body measurements.				
Weight	3	74.3		Kg.
Height	3	178.		Cm.
Surface area	3	1.85		Sq. m.
Temperature	3	37.2		°C.
Respiratory metabolism.				
CO_2	3	24.6		Gm.
O_2	3	23.4		"
H_2O	3	34.7		"
Calories, direct	3	65.9		Cal.
Calories, indirect	3*	66.3		"
Respiratory quotient	2	.82		
R. Q., severe diabetes	3	.697		
Per cent calories from protein fat and carbohydrate	2	17.		Per cent.

*This factor is for general use. For special cases it is more desirable to separate the protein and weigh it as such, or in cases where the nature of the protein is known to apply the proper conversion factor.

THE PERMEABILITY OF LIVING CELLS TO ACIDS AND ALKALIES.

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(Received for publication, August 10, 1916.)

Recent investigation has rendered it increasingly clear that one of the most powerful means of influencing metabolism is by changing the reaction of the protoplasm. In view of this, the penetration of acid and alkali into the cell has taken on additional interest.

Some of the most important contributions to this subject may be briefly mentioned. De Vries[1] found that the red sap of the cells of the red beet turns brown when placed in NH_4OH, but that on washing in water the red color again returns. Pfeffer[2] showed that the red sap of *Pulmonaria* petals, of the stamen hairs of *Tradescantia*, and of sections of beet root first becomes blue and then greenish in dilute NH_4OH, KOH, or K_2CO_3. Pfeffer[3] mentions that as a rule the original color returns when the tissue is well washed with water, while if the reagents are sufficiently dilute and the action not too prolonged, the cells remain living for a time.

Plant cells have not been utilized in recent investigations on the permeability of protoplasm to acids and alkalies, chiefly[4] because the blue anthocyan pigments of petals were considered not to be sensitive enough to weak acids (such as acetic) and also because of the fact that many plant cells are cuticularized and are therefore not readily wetted or penetrated by the solutions.

Bethe[5] found that neutral red could be introduced into cells as an indicator in the study of permeability. Warburg[6] in his work on the permeability of sea urchin eggs to alkalies, made use of this dye in staining the cells. Harvey,[7] in his studies on the penetration of cells to alkalies fou it difficult to secure animals whose cells contain a pigment that show marked color change on addition of alkali. In the absence of a suital pigment he introduced neutral red into the cells, a method which has b criticized recently[8] but which Harvey[9] has shown to be unaffected by p teins, lecithin, etc., in respect to sensitivity to different alkalies. It v found[9] that living cells would not stand staining with indicators of ac (as had been done previously for alkalies), so that organisms with natu pigment had to be found. Though many pigmented animals are kno to occur, those containing suitable indicators are quite rare.

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[1] De Vries, H., *Arch. Néerland. sc. exact. et nat.*, 1871, vi, 124.

[2] Pfeffer, W., Osmotische Untersuchungen, Leipsic, 1877, 140. Ruhland, W. (*Jahrb. wiss. Bot.*, 1909, xlvi, 1), has confirmed these observations.

[3] Pfeffer, The Physiology of Plants, Oxford, 2nd edition, 1903–06, i, 92.

[4] Harvey, E. N., *Science*, 1914, xxxix, 947.

[5] Bethe, A., *Arch. ges. Physiol.*, 1909, cxxvii, 219.

[6] Warburg, O., *Z. physiol. Chem.*, 1910, lxvi, 305.

While on the Barrier Reef expedition,[9] Harvey obtained *Sticho ananas*, the "prickly fish," which contains a natural indicator. The p mented testis of this form served as material for his study of the speed acid penetration of protoplasm. Crozier,[10] at the Bermuda Biologi Station, found *Chromodoris zebra*, a nudibranch, containing a suita indicator, which he utilized in studying the penetration of acids. Furt investigations at Bermuda have enabled Crozier[11] to describe the co changes of indicators derived from four pigmented animals, showing t natural indicators in animals are not quite so rare as had previously b supposed. As investigators have found it necessary to go to a consid able distance to secure naturally pigmented animals suitable for such st ies, it seems very desirable to ascertain whether plant tissues suita for such studies are not readily available.

The writer has found that many blue petals are admira adapted to the study of the penetration of acids and that ma red petals and other parts are well suited to the study of the pe tration of alkalies. In the course of these experiments the wri has made extensive use of the following: 1. Petals of *Browal speciosa*. 2. Petals of *Pelargonium*. 3. Perianth of "Queen the blue" hyacinth. 4. Root of red radish.

It is evident that in such investigations it is important compare the rate of penetration of (1) acids and alkalies of t same normal concentration as found by ordinary titration, a (2) acids and alkalies of the same hydrogen ion concentrat as found by means of the gas chain. Accordingly, a series 0.01 N acids and alkalies were prepared by ordinary titrat methods. A similar series of acids of 0.01 N hydrogen ion c centration and alkalies of 0.01 N hydroxyl ion concentration was made up by means of the gas chain. Approximately 50 cc. of each solution were run into each separate tumbler. The tumblers were ordinarily kept covered with glass plates. Blocks of rubber that had been boiled in water for several days were slit so that the plant material could be firmly held in the slit. After the insertion of the material into the rubber, the edge of the tissue was cut across smoothly by means of a sharp razor. This razor cut was made to allow the acid to penetrate through the uncuticularized walls of the cells, since it was found that penetration through intact cuticle is excessively slow. The cutting does not appear to injure in any way the cells in the neighborhood of the cut or to affect the color changes which were used as a criterion of penetration. The rubber cubes, below the surface of the solution, hold the material and should any air bubbles be attached to the tissue, they are readily removed with the help of a glass rod.

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[7] Harvey, *J. Exp. Zool.*, 1911, x, 507.

[8] McClendon, J. F., and Mitchell, P. H., *J. Biol. Chem.*, 1911–12, x, 45

[9] Harvey, *Am. J. Physiol.*, 1912–13, xxxi, 335.

[10] Crozier, W. J., *Science*, 1915, xlii, 735.

[11] Crozier, *J. Biol. Chem.*, 1916, xxiv, 268, 443.

The criterion of penetration was the first appearance of a very narrow, just distinguishable, band along the cut edge which was observed with the use of the daylight lamp.[12] If the cells are washed in distilled water when the first change of color appears, the normal color reappears. The primary object of the experiments has been to ascertain whether or not the same relative order of penetration found with animal tissues obtained also in the case of plant tissue. The experiments were run for as short a time as possible, usually less than ½ hour and seldom over 1 hour.

When a very weak acid is penetrating the cells very slowly, the indicator may be broken down before sufficient acid has entered the cell in order to cause a change of color in the indicator. When such cases occur they can be readily recognized by making an extract and then adding the indicator at various rates of speed to the acid in a test-tube. Short exposure of the plant material to the solutions decreases the possibility of errors due to pathological conditions.

In the following tables the acids are arranged in order of penetration, the most rapidly penetrating being placed first.

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[12] Crozier, *Science*, 1915, xlii, 764.

Each table gives the results of several (five to ten) serie experiments.

TABLE I.

Browallia Petals.

0.01 N total alkali (titration).
Ammonium hydroxide.
Sodium "
Potassium "

0.01 N total acid (titration).
Salicylic.
Benzoic.
Trichloroacetic.
Formic.
Hydrochloric.
Nitric.
Sulfuric.
Phosphoric.
Oxalic.
Tartaric.
Lactic. }
Citric. } Very slow.
Acetic. Extremely slow.

TABLE II.

Hyacinth Perianth.

0.01 N total acid (titration).
Benzoic.
Salicylic.
Trichloroacetic.
Hydrochloric.
Nitric.
Sulfuric.
Oxalic.
Formic.
Phosphoric.
Tartaric.
Lactic.
Citric. }
Butyric. } Very slow.
Acetic. }

TABLE III.

Pelargonium Petals.

0.01 N total alkali (titration).
Ammonium hydroxide.
Sodium hydroxide; potassium hydroxide.

Table I shows that the results with *Browallia* petals coincide with the results obtained with 0.01 N acids by Harvey and Crozier, working with animal tissues, except that in Harvey's experiments lactic and oxalic acids penetrated more rapidly than phosphoric. This difference is probably of little significance, since oxalic acid penetrates more rapidly than phosphoric into the perianth of the hyacinth (Table II).

Test-tube experiments with the natural indicators showed that on adding 0.01 N acetic or butyric acids the indicators broke down and changed color in such a way that they could not furnish a very satisfactory criterion of penetration. Acids which affect the indicators in this manner probably enter the cell slowly as a rule and may cooperate with other factors to decompose (or isomerize) the indicator.

The relative penetration of alkalies shown in Tables I and III agrees with the results of Harvey.

It is evident that as the hydrogen ion is the agent which causes the change of color in the cell (and doubtless is primarily responsible for the effect of acids on metabolism), we should test the penetration of the hydrogen ion in solutions which have the same hydrogen ion concentration. This, as far as the writer is aware, has never been done. Accordingly, a series of acids having a hydrogen ion concentration of 0.01 N and of alkalies having a hydroxyl ion concentration of 0.01 N, was prepared by the use of the gas chain. The results are shown in Tables IV, V, and VI.

TABLE IV.

Browallia Petals.

0.01 N hydrogen ion concentration (gas chain).

Acetic.	
Formic.	
Lactic.	
Trichloroacetic.	Approximately all the same.
Sulfuric.	
Oxalic.	
Nitric.	
Hydrochloric.	
Tartaric.	
Phosphoric.	
Citric.	

TABLE V.

Hyacinth Perianth.

0.01 N hydrogen ion concentration (gas chain).		0.01 N hydroxyl ion concentration (gas chain).
Acetic.		Ammonium hydroxide.
Formic.		Sodium "
Lactic.		
Oxalic.	Approximately all the same.	
Tartaric.		
Phosphoric.		
Hydrochloric.		
Citric.		
Sulfuric.		
Nitric.		
Trichloroacetic.		

When we compare the results obtained by making 0.01 N solutions by titration (Tables I, II, and III) with the results obtained by making solutions of 0.01 N hydrogen ion concentration by the use of the gas chain (Tables IV and V), we see striking differences. The most interesting is the fact that acetic jumps from the bottom to the top of the list; a similar change is seen in the position of lactic acid while a much smaller change occurs in the position of formic acid.

This is exactly what would be expected if the change in the color of the cell depends primarily on the penetration of the hydrogen ion. For when the solutions are made up, as in Tables IV and V, the hydrogen ion concentration of acetic acid is increased (as compared with the solutions in Tables I and II) more than the hydrogen ion concentration of lactic or formic, and consequently it ought to change its position on the list more than either lactic or formic.

Formic acid (ionization constant 0.0214) dissociates more than lactic acid (ionization constant 0.0138) and the latter more than acetic acid (ionization constant 0.00187), so that in a 0.01 N solution of formic acid (made by titration) there is a much higher concentration of hydrogen ions than in 0.01 N lactic acid and 0.01 N lactic acid has a greater hydrogen ion concentration than 0.01 N acetic acid. Hence we should expect 0.01 N formic to show greater penetrating power than 0.01 N lactic, and 0.01 N lactic greater than 0.01 N acetic, when the solutions are made up by titration as in Tables I, II, and III.

If the concentration of the hydrogen ion were the only factor governing penetration, we should expect all the acids in Tables IV and V to penetrate at the same rate. As a matter of fact, acetic penetrates more rapidly than formic and the latter more rapidly than lactic while the rest go in more slowly than lactic. The reason for these differences must be left to further investigation.

Similar considerations apply to the penetration of alkalies. If the hydroxyl ion concentration were the only factor governing the penetration of alkalies, we should expect both the alkalies in Table V to penetrate at the same rate, but we find that ammonium hydroxide penetrates more rapidly than sodium hydroxide.

An apparent basis for comparing the rate of penetration of hydrogen and hydroxyl ions is afforded by Table VI. The material (root of red radish) shows a change whether placed in acids or in alkalies, and hence we may roughly compare the penetration of the two. This, however, is of doubtful significance because we are unable to say whether the changes in color produced by the acid and alkali correspond to equal changes in hydrogen ion concentration.

TABLE VI.

Root of Red Radish.

Concentrations expressed as normal concentrations of hydrogen or hydroxyl ion (gas chain).

	N.
Series A.	
Acetic	0.0022
Hydrochloric	0.003
Sodium hydroxide	0.002
Series B.	
Acetic	0.0012
Hydrochloric	0.0013
Sodium hydroxide	0.001
Series C.	
Acetic	0.00045
Hydrochloric	0.00047
Sodium hydroxide	0.0008

SUMMARY.

1. Plant tissues containing natural indicators are admirably adapted for studying the penetration of acids and alkalies.

2. The relative rate of penetration of the hydrogen ion in 0.01 N solutions (made by titration) of acids and alkalies is practically the same in the plants studied as that found by Harvey and Crozier for animals.

3. Very different results are obtained when the solutions are made up with a hydrogen ion concentration of 0.01 N (by the use of the gas chain). We then find, for example, that acetic acid is at the top instead of at the bottom of the series (some other acids also change their position). This may be explained in part by the dissociation of the acids concerned.

THE ACIDITY OF PLANT CELLS AS SHOWN BY NATURAL INDICATORS.

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(Received for publication, August 14, 1916.)

The importance of ascertaining the actual reaction of protoplasm has become more and more evident with the progress of investigation. In view of this, the writer has endeavored to discover to what extent natural indicators, found in living cells, can be utilized to determine their reaction.

The value of this method is due to the fact that indicators show the actual rather than the total acidity, and it is the actual acidity which is of chief importance for life processes. The writer has found[1] that in plant cells there is no constant relation between the two.

The total acidity includes both the undissociated and the dissociated hydrogen ions, while the actual acidity depends only on the latter and is conveniently designated by the P_H^+ numbers of Sörensen, which are the negative common logarithms of the numbers expressing hydrogen ion concentration. Thus if the hydrogen ion concentration is $1 \times 10^{-7.5}$ N, the P_H^+ number is 7.5.

Before describing the results it may be of interest to mention briefly some previous contributions to the subject.

It is stated by Pfeffer[2] that the red color of rose petals, beet roots, etc., indicates acidity, while the blue coloration of the bluebell and hyacinth is taken as an indication that these cells are neutral or slightly alkaline. He also states that treatment of the cells with acid or alkali may change the reaction without at once killing the cells and that upon thorough washing, the color of the normal reaction returns. Flowers of *Pulmonaria* have been observed to change from red to blue as they grow older, indicating a change in the reaction. Kraemer[3] has succeeded in modifying the color of flowers by adding reagents to various kinds of soil. (When cut flowers and plants were supplied with dyes or color substances, only a few were carried as high as the flower, indicating that only certain substances could be taken up by the plant and exert an influence on the coloring matter in the flower.) Blue chicory flowers have been observed by Kastle and Haden[4] to change from blue to white and finally to brown, with practically complete destruction of the flower pigment, due in part to variations in acidity and in part to the oxidase action which completely oxidizes and destroys the flower pigment. The red, blue, and colorless solutions of the blue chicory flower pigment were found to be acid to phenolphthalein and even the yellowish green solutions did not always give an alkaline reaction with phenolphthalein. The color change of phenolphthalein is, according to Sörensen's table, colorless to red, $P_H + 8.3$ to $P_H + 10.0$, which indicates that a solution may still be colorless to phenolphthalein and be alkaline.

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[1] Haas, A. R., *Bot. Gaz.* (in press).

[2] Pfeffer, W., Osmotische Untersuchungen, 1877, 140; *Untersuch. Bot Inst. Tübigen*, 1886, ii, 293.

The experiments of the writer were carried on with a variety of anthocyan pigments,[5] especially those of petals. Buffer solutions from P_H^+ 1 to P_H^+ 13 were made up by means of the gas chain, so that the hydrogen ion concentration of each solution was accurately known. Aqueous or alcoholic extracts of the pigments were made by rapidly macerating the petals in a mortar with the solvent. The activity of enzymes in destroying the pigments required that in many cases the buffers be handled rapidly. The extracts were made as concentrated as possible and to each buffer solution the same number of drops of the extract of pigment was added. The resulting colors are given in the following tables. Rapid decolorization of the pigment is indicated by a vertical line. Petals are used unless otherwise stated.

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[3] Kraemer, H., *Science*, 1906, xxiii, 699; 1909, xxix, 828.

[4] Kastle, J. H., and Haden, R. L., *Am. Chem. J.*, 1911, xlvi, 315.

[5] It is chiefly to Willstätter and his students that we owe our knowledge of the nature of anthocyan. Willstätter, R., *Ber. chem. Ges.*, 1915, xlvii, 2831; *Ann. Chem.*, 1914, cdviii, 1; *Sitzungsber. Akad. Wiss. Berlin*, 1914, xii, 402; *Ann. Chem.*, 1913, cdi, 189. See also Crocker, W., *Bot. Gaz.*, 1916, lxi, 349, and Wheldale, M., *J. Genetics*, 1914, iv, 113; 1910–11, i, 134; *Proc. Roy. Soc., Series B.*, 1909, lxxxi, 44; *Proc. Cambridge Phil. Soc.*, 1909, xv.

TABLE I.

Flowers.

P_H^+	Sweet pea. Lavender. 95 per cent alcohol extract.	Pansy. Blue. 95 per cent alcohol extract.	*Primula obconica.* Pink (flowers turn blue, then white in fading). Water extract.	"Queen of the blue" hyacinth. Blue. Water extract same as alcohol extract.	Sweet pea. Pink. Water extract.	Pansy. Blue. Water extract.
1	Pink.	Rose.	Rose.	Pink.	Pink.	Rose.
2	"	"	"	"	"	"
3	Lavender.	Pink.	Pink.	"	"	"
4	Pale lavender.	Violet.	"	Pink-blue.	"	"
5	"	Blue.	Red-violet.	Blue.	"	Blue.
6	"	Blue-green.	Violet.	"	Pink-violet.	Blue-green.
7	Green.	"	Blue.	Blue-green.	Blue.	"
8	"	Green.	Green.	Green.	Blue-green.	"
9	"	Yellow.	"	"	Green.	Green.
10	"		Green to yellow.	"	"	"
11	"				"	"
12					Yellow.	Yellow.

TABLE II.

P_H^+	*Cichorium intybus.* Blue. Water extract.	Violet. Blue. Water extract.	*Scilla.* Blue. 50 per cent alcohol extract.	Hyacinth. Red. 95 per cent alcohol extract.	*Primula chinensis.* Red. 95 per cent alcohol extract.	*Primula chinensis.* After red petals had changed to blue. 95 per cent alcohol extract.
1	Pink.	Pink.	Pink.	Red-orange.	Not determined.	
2	"	"	"	"	"	
3	Blue.	"	"	Light orange.	"	
4	"	"	Pink to violet.	Pink.	"	
5	"	Violet.	"	"	Pink.	P_H^+ 6.23 violet.
6	"	Blue.	Blue.	"	"	P_H^+ 6.5 blue by reflected light.
7	Violet.	"	"	Violet.	Blue.	P_H^+ 6.97 brown-green.
8	Blue-green.	Blue-green.	Green.	Green.	Blue-green.	P_H^+ 7.38 green.
9	Green.	Green.	"	"	Green.	P_H^+ 8.04 green.
10	"		"			

TABLE III.

P_H^+	*Pelargonium.* "Paul Crample." Pink. 95 per cent alcohol.	Hyacinth. Dark blue. 95 per cent alcohol.	*Browallia speciosa.* Blue. 95 per cent alcohol.	Blue violet. Extracted with 95 per cent alcohol; filtered; evaporated to dryness; dissolved in water as indicator.		Blue pansy. Water extract of indicator obtained in same way as for blue violet.	
					*		†
1	Orange-yellow.	Red.	Red.	Red.	Red.	Red.	Red.
2	"	"	"	Rose.	Rose.	Rose.	Rose.
3	Pink.	Pink.	Pink.	Pink.	Pink.	Pink.	Pink.
4	"	"	"	Pink-violet.	Pink-violet.	Violet.	Violet.
5	"	Blue.	"	Blue-violet.	Blue-violet.	Blue-violet.	Faint blue.
6	"	"	Blue.	Blue-green.	Green.	Purple.	Green.
7	"	Blue-green.	"	"	"	Blue.	"
8	Blue.	Green.	Blue-green.	Green.	"	Blue-green.	"
9	Violet.	"	Green.	Green-yellow.	Yellow.	Green.	
10	"		"	"	"	Yellow.	
11	Green.		"	Yellow.	"		
12	"		Yellow.	"	"		

*After standing in buffer solutions 15 hours.

†After standing in buffer solutions several hours.

TABLE IV.

P_H^+	Blue violet.*	Red cabbage leaves. Purple. Water extract.	Cranberry juice on paper. Red. When dry, touched with drop of each buffer solution.	Cranberry peelings boiled with water to extract the pigment. Red.	Radish root. Red. 95 per cent alcohol extract.	Red beet root juice. Water extract.
1	Red.	Rose.	Pink.	Reddish orange.	Orange.	Rose.
2	Rose.	"	"	"	"	"
3	Blue.	Pink.	"	Rose.	Pink.	Red.
4	"	"	Violet.	"	Less pink.	Becoming gradually darker after $P_H^+ 7$ until at P_H^+ 10 and 11 it is of a violet or bluish red color.
5	"	Violet (pink-blue).	Blue.	"	Less pink than P_H^+ 4.	
6	Blue-green.	Violet (pink-blue).	Blue-green.	Violet.	Less pink than P_H^+ 5.	
7	Green.	Purple.	Green.	Both P_H^+ 7 and P_H^+ 8 pass through pink-violet to bluish, then turn brown. Very rapid changes.	Less pink than P_H^+ 6.	
8	"	Blue-green.	"		Blue at H ion concentration of 4×10^{-8}.	
9	"	"	"		Blue-green to green.	
10		"	"	Reddish brown.	Green.	
11		"	(Pink.)	Pink.	"	
12		Green-yellow.		Brown.	Yellow.	Orange-brown.
13		Golden "		Green.	"	Yellow.

*Extracted with 95 per cent alcohol—$CHCl_3$; evaporated to dryness; the residue dissolved in water; water extract shaken with CCl_4; evaporated to dryness; redissolved in water for use as indicator; colors stable in buffer solutions after standing 4 days.

The colors were observed under a light of constant and excellent quality from a recently described lamp.[6] It was not considered desirable to attempt a more accurate description of the colors because of the considerable degree of variation dependent upon the strength of the indicator. In radish the indicator was very dilute so that shades of red varied to shades of pink. Some petals, such as those of pansy and violet, contain very large amounts of anthocyan in comparison with others (such as light blue hyacinth, *Browallia speciosa*, etc.) and in extracts of the former the colors observed with buffer solutions more nearly match the normal color of the cells from which the indicator was extracted, as regards the intensity of the color. The number of drops of indicator and the number of cc. of buffer solution used were always such that the results for a single indicator are comparable at the various concentrations of hydrogen ion.

When crude extracts of the pigment are used, the indicator often decolorizes so quickly that it is necessary to filter as rapidly as possible (or the filtration may be omitted entirely if necessary). When extracts were somewhat purified the indicator remained more stable, even for long periods, which may indicate that in crude extracts the decolorization may be due (in part at least) to the action of enzymes. Some indicators when added to buffer solutions of hydrogen ion concentrations in the region of P_H^+ 4 to P_H^+ 6 rapidly decolorize, but upon the addition of very strong acid in sufficient quantity, the color characteristic of the indicator for acid solutions of P_H^+ 2 or 3 reappears. This may indicate that the colorless condition of the indicator may be due to an isomerization of the indicator or the formation of a leuko base.

The results showed very little difference whether crude alcoholic or aqueous extracts of the indicator were used. It seemed very surprising that cranberry juice indicator at P_H^+ 11 should give a pink color, but a redetermination of the buffer solution on the gas chain showed the previous determination to be accurate, and when the indicator was dried on paper it was shown that a drop of buffer solution of a strength of P_H^+ 11 gave a pink color. Red beet juice may be bluish red when slightly alkaline. Hence the pigments of some plant cells (when the indicator is used *in vitro*) may assume a more or less reddish color when alkaline.

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[6] Luckiesh, M., *Science*, 1915, xlii, 764.

It is evident from a consideration of the results that the reaction of the cells studied ranges from about P_H+ 3 to about P_H+ 8.

In a number of cases the color of the flower changes as the flower fades, thus indicating a change in reaction which accompanies the death of the cells. When *Browallia* petals are just expanded they are blue, but when past their maturity they may become somewhat blue-green in certain parts. *Primula obconica* at first has pink petals but later the color changes to a blue and then may become white. In comparison with *Primula obconica* it is well to consider *Primula chinensis*. The petals of *Primula chinensis* are normally red but later become blue, which color is permanent. This blue color results similarly when the petals are removed while red and are placed in a desiccator.

These two *Primula* flowers, by the change of color in the petal cells, show a decrease in acidity when the cells become old or die. The data further indicate that the cells of *Primula chinensis* are still very slightly acid when the cells die. Such small amounts of acid cannot be detected by ordinary titration methods. If we call the buffer solutions acid up to P_H+ 7, neutral when at P_H+ 7, and alkaline when higher than P_H+ 7, it is evident from the experiments that it is unsafe to call cell sap acid when red, neutral or alkaline when blue, and markedly alkaline when green, unless the color changes of the particular pigment are first studied by some method such as that of using buffer solutions of known hydrogen ion concentration.[7]

Mann considered that the coloration of the anthocyan in the seed coat of cow-peas[8] indicated acid when rose or purple and alkali when blue or black, and that both the alkaline- and the acid-reacting anthocyanine may occur in the same cell. The writer was unable to find in microtome and hand sections of cow-peas (of different colors), fresh from immature and mature pods, any cases where the same cell contained both red and blue pigments before drying. The cells were mounted both dry and in water. It may be added that if the color comes out in water it indicates that the cells are dead.

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[7] Crozier, W. J., *J. Biol. Chem.*, 1916, xxiv, 443.

[8] Mann. A., *J. Agric. Research,* 1914, ii, 39.

In this connection the writer has made observations on several tropical flowers (*Bromeliaceae*) which have a blue pigmented portion merging into a red pigmented part. In fresh material no cells were found to contain both red and blue colors unless the section became dried, in which case one end of the red cell became blue and the blue color progressed along the cell until all of the cell was blue. Fresh material showed cells containing red pigment to adjoin cells containing blue pigment. The fact that blue cells never became red indicates that the blue pigment represents a decrease in the acidity of the cell sap. At P_H^+ 8 the pigment extracted from both red and blue parts gave a bluish green coloration with buffer solutions, while at P_H^+ 9 the pigment extracts gave a green coloration with the buffer solutions. It is evident, therefore, that the blue color of a pigment is insufficient as a criterion of the reaction of the cell unless the pigment is first calibrated by solutions of known hydrogen ion concentration.

SUMMARY.

1. In view of the widely accepted idea that the reaction of living cells must be neutral or nearly so in order that life may go on in a normal manner, it is of interest to find normal cells in which the reaction of the cell sap is decidedly acid (P_H^+ 3).

2. The prevailing opinion that the blue color of living cells always indicates an alkaline reaction is erroneous. It is found to indicate a decidedly acid (P_H^+ 3) to neutral reaction (P_H^+ 7), or sometimes barely alkaline (P_H^+ 7 to 8).

3. A considerable change of reaction may occur in the cell as it dies (from P_H^+ 3 to P_H^+ 7).

THE INFLUENCE OF BILE ON AUTOLYSIS.

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While it is common knowledge that bile possesses very decided toxic properties, the literature on this subject leaves much to be desired in regard to the nature and mode of action of the active constituents. The substances which have been accredited as responsible for the toxicity are accordingly (1) the bile salts, sodium glycocholate and sodium taurocholate; (2) cholesterol; (3) bile pigments; and (4) lecithin. The possibility cannot be overlooked that the various groups of investigators, though apparently reporting conflicting results, are yet partially correct, depending upon the criteria used for their interpretations. It is quite probable that the different constituents of bile may act upon the various body structures quite differently, and thus be responsible for the diversity of opinion as to which is and which is not the toxic constituent.

It has long been known that bile and bile salts have a powerful cytolytic action (Rywosch (1)) though the mechanism of this action does not appear, to my knowledge, to have been clearly defined. It is with such a problem that this paper is concerned.

Small blocks of various organs were taken as soon as possible after killing an animal, placed in the freshly withdrawn bile, then allowed to stand in the incubator at 38°C. from 2 to 4 hours. The bile was then poured off, and the tissues fixed in Zenker's solution. Sections were prepared in paraffin and stained with hematoxylin and eosin. There is found to have occurred a very marked alteration at the periphery of the tissues, while the central portions are unchanged. In the peripheral margins, the nuclei have lost their capacity for staining with hematoxylin, even losing all evidence of their former position or form. Th cytoplasm takes the eosin stain, but most peripherally become pale or even disappears altogether, leaving merely the supporting connective tissues. Within, the nuclei are pyknotic, formin a narrow zone of dense blackened nuclei just without the centra normally appearing, unaffected area.

The depth of the changed cortical area depends upon severa factors. These factors appear to be the penetration of the cytolytic agent, the character of the tissue, and the alteration produce in the affected zone. In regard to actual depth of action, it ma be stated that nuclei in several specimens of series running for hours had completely disappeared throughout in tissues 0.3 t 0.4 cm. in diameter. Consequently I lost many specimens because they were too thin, or were left too long in the incubator before I was fully aware of the rapidity of the reaction.

The question then arose, what is the significance of this marke cell change? It is generally stated that bile or its salts have i themselves no digestive action, except perhaps a slight actio on carbohydrates, but here is evidence of marked alteration even of protein material. If the cytolytic action is a solven process, it should act upon fibrin and other pure proteins, whic it does not do. The reasonable explanation appeared to b that the autolytic processes of protoplasm are markedly accelerated by bile or certain of its constituents, and consequently i was found that no alteration could be demonstrated in the tissues, if previously to incubation in the bile they were killed b immersion for a minute in boiling water. This condition obviousl is now comparable to the negative results obtained on precipitated proteins. Here the conditions necessary for autolyti processes are destroyed and hence the bile cannot exert its cytolytic action. Further evidence of this view of the action of bil is obtained in the following experiment.

Tissues were immersed in bile, and placed, respectively, in the incubato at 38°C.; on the laboratory table at 25°C.; and in the ice box, at 0°C After 2½ hours the tissues were dropped into Zenker's fluid and finall sectioned. On examination it was found that practically no action ha occurred in the cold specimen, considerable in that at 25°C., and mos in that kept at the optimum temperature of 38°C. This follows the la of enzyme action with respect to the influence of temperature. Furthe evidence of autolysis is the fact that those tissues most rapidly autolysin post mortem are those that are most affected by bile salts, namely, in order, adrenal, spleen, liver, thyroid, heart muscle, smooth muscle, voluntary muscle, and lastly, connective tissue, which is not affected.

Each block of tissue presents its own control, since the interior is unaltered, though under the same temperature influence. However, separate controls were studied in Ringer solution, in chloroform-Ringer, and in serum. In none of these instances did there occur the slightest evidence of autolysis during the time of incubation, 2 to 4 hours. In serum and alkaline Ringer solutions no autolysis could be expected, since serum (5) is known to be protective against autolysis and against hemolysis of erythrocytes, and alkalies (6) are also known to inhibit autolysis. According to some investigators chloroform-Ringer (7) should have given positive evidence of autolysis, but these authors allowed a longer duration of autolysis than that used in my bile experiments. Hence, while chloroform may be a real accelerator of autolysis, it is extremely weak compared to bile or bile salts. The same is true for acid solutions (8) which have been found to accelerate autolysis, but they are also extremely weak compared to bile action. As an illustration of this, on comparison of the effect of 5 per cent bile in Ringer solution on a specimen of fresh thyroid gland it was found to be very much greater than the effect of 0.05 N or 0.025 N HCl on another specimen of thyroid gland. It will be recalled that the optimum concentration of acid for autolysis (Bradley (8)) is near 0.05 N acid. I have repeatedly found that diluted bile is considerably less active than whole bile. While I cannot say, from the criterion employed, that 5 per cent bile is one-twentieth as active as whole bile, yet the effect of the optimum concentration of acid is very much below this 5 per cent bile; consequently the differences in activity are in all probability greater than the rough estimate I have given.

End-points are not readily determinable by this technique, consequently only alterations in coagulable proteins that are subject to morphological differentiation as occurring in cell structures are used as criteria of rate and degree of autolysis.

In order to ascertain, if possible, the relative importance of the constituents of bile, some analytic experiments were carried out. Gall bladder bile is known to vary considerably in its quantitative relations, consequently it was deemed advisable to determine the amount of bile salts in a particular sample of bile and then make up solutions of commercial bile salts to the corresponding concentration.

Sheep bile was obtained fresh from a near-by abattoir. A portion was placed in the ice box till analysis and special treatment of the other portions were completed. On analysis, according to the method given by Hoppe-Seyler (9), this specimen of bile was found to contain 10.44 per cent of bile salts. This figure was used as a basis of subsequent preparations in this experiment.

To a third portion an equal volume of animal charcoal was added and it was then evaporated to near dryness over the water bath. This was extracted repeatedly with alcohol and each extraction filtered. The pale yellow extract was then evaporated to dryness over the water bath, when the residue was taken up by distilled water. By this means, most of the pigment and all of the mucin were removed.

For autolytic tests, all solutions, unaltered bile, decolorized mucin-free bile, and solutions of sodium glycocholate and of sodium taurocholate were made up to 40 per cent of the original concentration as determined. Thus whole bile was made up to 40 per cent, as was the decolorized mucin-free bile. Sodium glycocholate[1] was made up to 40 per cent of the original salt concentration of bile, which made a 4.18 per cent solution. The sodium taurocholate[1] was marked "47.5 per cent," consequently sufficient salt was weighed out to make a 4.18 per cent solution. Thus I was able to compare the action of equally concentrated solutions of bile, of decolorized mucin-free bile, sodium glycocholate, and sodium taurocholate.

Approximately equal sized portions of absolutely fresh rabbit liver were placed in each of the four solutions and incubated at 38°C. for 2 hours. The tissues were then killed by Zenker's fixative.

The least penetration, as measured by the position of the zone of pyknotic nuclei, occurred in the specimen in sodium glycocholate, though the cytoplasm of the marginal cells had undergone considerable solution. Sodium taurocholate penetrated to a greater depth, equal to that of bile solutions, but no solution of cytoplasm appears to have occurred. The decolorized mucin-free bile was practically similar in action to the solution of sodium taurocholate, while the greatest amount of solution of cytoplasm occurred in the specimen immersed in unaltered bile.

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[1] Merck and Co.

In this experiment it is found that whole bile has a total activity greater than that produced by corresponding concentrations of the bile salts[2] taken separately. It is also greater than that of bile treated with animal charcoal and alcohol. The character of the active constituents lost by this process I have not determined. The statement can be made, however, that whole bile contains a mixture of active constituents whose total activity is not equaled by bile treated with animal charcoal and alcohol, nor by corresponding concentrations of separate solutions of either of the bile salts. Further, the two bile salts appear to behave somewhat differently, the sodium glycocholate having the greater solvent effect on cytoplasm. This may be taken to indicate a degree of specificity of particular accelerators for autolytic processes.

No precautions were taken to prevent bacterial action. The reaction is much too rapid to be ascribed to bacterial decomposition since in control experiments without bile no comparable changes are produced. Sellards (5) found necrosis to occur in the salivary glands in the living animal when injected aseptically with bile or bile salts; and furthermore, the results of the experiments of Bunting and Brown (11) on necrosis following intraperitoneal injections of bile are not likely to be explained by bacterial contamination. Boiling the bile previous to its application to tissues does not diminish its activity, while no evidence of activity is found when boiled tissue is treated with unboiled bile.

The conclusions drawn from the work here presented are the following.

1. Bile is a powerful cytolytic agent.
2. This cytolysis is produced by virtue of the co-enzyme or activating action of the constituents of bile on the autolytic enzymes or processes.

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[2] According to Long and Johnson (10) the various commercial preparations of bile salts are subject to rather wide variations in actual concentration of the pure bile salts; consequently the comparisons will necessarily be held in some reserve.

3. Whole bile is more powerful though less penetrating than corresponding concentrations of either of the bile salts.

4. Bile boiled down with animal charcoal and treated with alcohol has lost a portion of its activity.

5. The maximum effects of bile or bile salts are many times greater than the effects of the optimum concentration of acids.

6. Sodium glycocholate appears to affect cytoplasmic material more than does sodium taurocholate, though both affect nuclear material about equally.

7. These facts, correspondingly, have to be considered in any study of the toxicity of bile, and may center attention on alterations in cellular metabolism produced in such intoxications, and furthermore, this agent may be of service in the study of the fundamental factors of autolysis.

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THE RATE OF UREA EXCRETION.

II. THE RATE OF EXCRETION OF ADMINISTERED UREA IN YOUNG HEALTHY ADULTS ON A CONSTANT DIET.

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This study of the rate of excretion of urea administered to young healthy adults who were on a constant diet was carried through in order to obtain detailed information as to the range of variation in the function of the normal kidney under uniform conditions. The work of Schlayer and others on water and chloride excretion by the kidneys has been interpreted as indicative of a wide variability in kidney action even under the same conditions. This variation is assumed to arise from the inconstancy of what is termed the *Reizzustand* of the organ. On the other hand, Ambard believes that the kidney manifests no intrinsic variability in function. He maintains that if the concentration in the blood and in the urine of the substance excreted is known, the action of the normal kidney may always be predicted with mathematical accuracy.

We have found in the literature only two single experiments in which the rate of urea excretion after urea administration has been determined in man by dependable methods. Wolf[1] and Cathcart and Green[2] state that urea taken by mouth is quantitatively excreted within 24 to 48 hours, and Wolf showed that the rate of excretion was greatest in the first few hours after the ingestion of the urea.

Achard and Paisseau,[3] McCaskey,[4] and Pirondini[5] showed that results which were of value in the detection of functional abnormalities in renal disease may be obtained even when the unreliable hypobromite method of urea estimation is used.

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[1] Wolf, C. G. L., *Biochem. Z.*, 1912, xl, 234.

[2] Cathcart, E. P., and Green, H. H., *Biochem. J.*, 1913, vii, 1.

[3] Achard, C., and Paisseau, G., *Compt. rend. Soc. biol.*, 1904, lvi, 894.

[4] McCaskey, G. W., *Med. Rec.*, 1914, lxxxv, 507.

[5] Pirondini, E., *Policlinico Sez. Chir.*, 1915, xxii, 259, 326, 398.

The general plan we adopted was to measure the rate of excretion during a fore-period of 3 days, and during a period of 3 successive days on which 20 or 40 gm. of urea were administered. The urine was collected every 4 hours during the day, and a 12 hour collection was made at night.

The subjects of these experiments were instructors, internes, and students. The ages were between 20 and 35 years. All were in apparent health.

Methods of Urea Estimation.

We had already planned and commenced this work, using Folin and Farmer's microchemical methods of nitrogen determination in the blood and urine, before Marshall's description[6] of the urease method appeared. But after we had confirmed the accuracy of his method we used it exclusively, both for urine and blood analyses. It is unnecessary to give any of our figures in substantiation of the soundness of the principle of the method, since this has already been amply established. But we had to discard our earliest results until we had adopted the two following modifications in working with urine:

1. At first we used 10 per cent thymol in chloroform as a preservative for the urine. We noted occasionally that the amount of urea found was less than the expected quantity, in spite of the fact that duplicate estimations checked perfectly. These were always cases in which the urine was alkaline in reaction. Part of the urea had already been converted into ammonium carbonate. In any neutral or alkaline urine this change takes place with great rapidity, at least in any laboratory where urine is a material which is commonly worked with. It is due to the action of urease-containing organisms. The usual urinary preservatives, such as thymol, chloroform, and toluene, all of which we tried, are not always able to prevent their action. Acting on Armstrong and Horton's[7] finding that the action of urease is absolutely inhibited by any concentration of H ions much above that found at the neutral point for litmus, we thereafter had the urine passed directly into vessels containing 20 cc. of N H_2SO_4 for each 4 hour collection. Except in the case of patients with an infection of the urinary passages, in which some urease-producing organism is present which decomposes the urine before it leaves the bladder, we have never since then had any trouble from this cause.

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[6] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xiv, 283.

[7] Armstrong, H. E., and Horton, E., *Proc. Roy. Soc.*, 1912, lxxxv, 109.

2. Another very necessary modification is the neutralization of the urine before adding the urease extract. Some urines are sufficiently acid to destroy such a considerable part of the ferment before it can commence to form ammonium carbonate that the urea present is not completely converted. We discovered this early in our work, since we had adopted the plan of running duplicate estimations to one of which a known amount of urea was added. An incomplete recovery of this urea, when a corresponding quantity of urea in water was completely accounted for, could not be due to anything but some inhibitory substance in the urine. This inhibition only occurred with acid urines and was completely removed by bringing the urine to the neutral point for rosolic acid. This indicator was chosen, since it changes color just about the point of absolute neutrality.

All the estimations reported here were carried out after the adoption of these two precautions—the collection of the urine in N H_2SO_4, and its subsequent neutralization to the neutral point for rosolic acid.

The details of the technique used for urine were as follows: The specimens of urine were measured in graduated cylinders. They were then poured into volumetric flasks and diluted to the mark with water, so that about 10 cc. of 0.1 N HCl would be required to neutralize the ammonium carbonate formed from 5 cc. of urine. This is accomplished by diluting the 4 hour specimens of the first 3 days to 500 cc., and the night urine to 1,000 cc. On the 4th, 5th, and 6th days when urea is taken, the 4 hour specimens were diluted to 1,000 cc. and the night specimen to 2,000 cc. This high and nearly uniform dilution makes the end-points much easier to recognize.

5 cc. of urine were pipetted from each specimen into each of three flasks, and about 100 cc. of distilled water and two drops of rosolic acid were added to each. All were brought to a first faint red color by adding the same amount of 0.1 N NaOH. 2 cc. of an aqueous extract of freshly powdered soy bean was pipetted into two of the flasks, and after the addition of a few drops of toluene and a known quantity of urea to one, they were corked and left until the following day. To the remaining flask, methyl orange was added and it was titrated at once with 0.1 N HCl until the first tinge of pink developed after the disappearance of the red color due to the rosolic acid. The amount of 0.1 N HCl required was noted, and to it was added the acid required to bring 2 cc. of the urease extract in 100 cc. of water to the same color. Next morning, using the same indicator, the two fermented flasks were titrated to the neutral point with 0.1 N HCl. The acid was run in quickly until the red of the rosolic acid had disappeared. Thereafter it was added slowly, watching for the appearance of a cloudiness which results from the precipitation of some substance in the soy bean extract. This occurs just before the end-point is reached and tends to obscure it somewhat. We found that we were helped in deciding as to the exact end-point if we observed the color of an added drop of methyl orange before it diffused through the fluid. A deep reddish brown color is given if sufficient acid has already been added. The flasks should not

be employed for any other purpose. Even slight traces of silver or copper salts interfere markedly with the action of the ferment. Solutions of urea of known concentration prepared from urea purified by repeated crystallization were estimated each day to check the activity of the soy bean extract.

The Diet.

The diet was constant as regards nitrogen, but provision was made for some degree of elasticity in the amount of carbohydrate taken. Each meal was well within the capacity of a moderate appetite. After it was finished a nitrogen-free cornstarch paste was served. When taken with a spoonful of jam and the small amount of cream which was provided, this made a pleasant dessert. If the subject were not hungry he could leave it out, taking only the cream, and if he happened to be hungry he could take it all. As a matter of fact all the subjects took some of this cornstarch, and nearly all of them took it all, so that the variation in water intake from this cause was not great.

The meals at 8 a.m., 4 p.m., and 8 p.m., were similar and consisted of one egg, 50 gm. of bread, 10 gm. of butter, and 5 gm. of cocoa mixed with 240 cc. of milk. The 12, noon, meal contained 150 cc. of Campbell's tomato soup, neutralized with $NaHCO_3$, and heated with an equal quantity of milk, 50 gm. of bread, 10 gm. of butter, 50 gm. of potatoes, and one egg. 180 cc. of water were taken with this meal, and this was the only water allowed, as such. A little NaCl was given with the egg.

The total water content of the diet (including the 180 cc. of water and the water in the cornstarch) was about 1,710 cc. The protein content was 75.9 gm., the fat 120 gm., and the carbohydrate 220 gm., including the cornstarch.

We are much indebted to Miss Sloan, the dietitian of the Stanford Medical School, for the care she took in weighing and preparing this food.

The Total Urea Excretion.

In Table I, the averages and limits of variation in the total urea excretion for each day of the diet are given. These are compiled from the results obtained in thirty individuals who took 20 gm. of urea on the 4th, 5th, and 6th days of the diet.

The effect of the variable quantities of nitrogenous food taken before the diet was begun does not last beyond the 1st day of the diet. In Table I, the averages for the 2nd and 3rd days are practically identical. In cases to whom no urea was given, the average for the 2nd day did not differ appreciably from those of the subsequent days. Thus in six subjects on the diet, who took no urea, the average 24 hour urea excretion for 6 successive

TABLE I.

Averages and Limits of Variation of the Total 24 Hour Urea.

Fore-period.

1st day.			2nd day.			3rd day.		
Average.	Highest.	Lowest.	Average.	Highest.	Lowest.	Average.	Highest.	Lowest.
17.40	22.8	12.9	15.78	19.2	10.7	15.45	19.5	9.4

Urea period.

20 gm. of urea at 8 a.m. each day.

4th day.			5th day.			6th day.		
Average.	Highest.	Lowest.	Average.	Highest.	Lowest.	Average.	Highest.	Lowest.
32.27	35.5	28.1	34.37	40.7	30.0	34.53	40.5	29.5

days on the diet amounted to 16.8, 15.7, 15.9, 15.9, 14.9, and 15.1 gm. With the diet we used, therefore, an equilibrium is reached on an average within 24 hours.

The averages during the urea period show that the 20 gm. of urea administered are not quite completely excreted. There is a retention of a few gm. of urea which can be accounted for if the diet is continued for another day without giving urea. It is also noteworthy that there is a slight rise in the average excretion on each day during the urea period.

The range of variation amounts to about 10 gm. a day and is quite as large in the fore-period as in the urea period. The main cause of this wide variation becomes apparent when the data are arranged in order of magnitude. It is then seen that the higher values are derived throughout from one group of individuals, and the lower from another. In Table II all the urea excreted while the diet was taken with the exception of the urea of the first day is given for each individual.

The range of variation in Table II is due to some subjects having on every day of the diet a higher urea excretion than others. This explains the range of variation in Table I, for there the highest urea excretions for each day are derived almost without exception from the subjects BO, and Ad. who excreted more

TABLE II.

The Total Urea Excreted Excluding the 1st Day.

(Arranged in order of descending magnitude.*)

No.	Subject.	Urea.	No.	Subject.	Urea.	No.	Subject.	Urea.
		gm.			*gm.*			*gm.*
1	Bo$_1$	148.5	10	G	137.8	19	Ai$_2$	127.2
2	Ad	142.9	11	S$_1$	137.2	20	Boy$_1$	126.4
3	C	140.8	12	F	136.1	21	Br$_2$	126.3
4	Br$_1$	140.2	13	Cr	134.9	22	B	125.6
5	Wa	138.9	14	J	133.4	23	Add	124.5
6	P$_2$	138.1	15	S$_2$	131.2	24	K$_2$	124.2
7	K$_1$	138.0	16	P$_1$	130.7	25	We	123.8
8	D	138.0	17	Je	127.8	26	Ai$_1$	121.2
9	Boy$_2$	137.9	18	K$_3$	127.6	27	M	110.4

* The totals of Bo$_2$, O, and W cannot be given, since one collection of urine was lost in each of these cases.

than 140 gm. of urea in 5 days, and the lowest urea excretions for each day come from the subject M, who excreted only 110 gm.

These constant differences in the level of urea excretion are certainly not to be ascribed to corresponding differences in the capacity of the kidneys of these subjects in excreting urea. If that were the case, the subject M would have had a high concentration of urea in his blood and tissues on account of very marked urea retention. But as a matter of fact, the blood urea concentration of M on the 3rd and 6th days of the test was only 0.0156 and 0.0558 per cent, while the blood of BO$_1$ on these days had a concentration of 0.0261 and 0.0672 per cent. The subject M, who excreted the lowest amount of urea, had on the 3rd day of the diet the lowest blood concentration we have observed.

The cause of these different levels in urea excretion lies in the existence of different levels in the rate of protein catabolism. Although M took as much protein as BO$_1$, he did not excrete so much urea, because in his case presumably the amino-acids were in great part utilized in protein synthesis. In the case of BO$_1$ on the other hand, a much larger proportion was deaminized, with the resulting formation of larger amounts of urea.

The variable factor introduced by the existence of these different levels of protein catabolism in different individuals must be eliminated if we wish to compare the work of their kidneys in excreting preformed urea. This will be accomplished if we accept the average excretion of the 2nd and 3rd days of the fore-period as a measure of the level of protein catabolism for each subject, and subtract this value from his urea excretion on the days when urea was given. The difference gives the rate of excretion of the preformed urea which was given at the same time and in the same manner to all. For convenience we have termed the results of this subtraction "administered urea excretion."

The Degree of Variation in the Rate of Excretion of Administered Urea.

In Table III the rate of excretion of administered urea thus calculated is given for each subject for each day of the urea period along with the average excretion of each subject for that whole period. The average excretion of the fore-period is also given. Each column is arranged in order of descending magnitude, so that some idea of the degree of dispersion may be obtained at a glance.

Before the significance of these figures in Table III as indicative of functional variation in the work of the kidney can be appreciated, it is necessary to obtain some conception of the variation which might be introduced through the error in our method of calculating the amounts which we have termed administered urea excretion. For, although by taking the average excretion of the fore-period as representing the level of protein catabolism and subtracting it from the total excretion after urea administration, we have eliminated an inconstant factor—the variable rate of protein catabolism in different individuals—we cannot thus obtain a mathematically accurate representation of the rate of work of the kidney in excreting the administered urea. For there are not only differences in the rate of protein catabolism in different individuals; there are also smaller though not inconsiderable fluctuations from day to day in the same individual. These individual variations are not taken account of in our figures, since they are obtained by a method which assumes that the amount of urea obtained on the 2nd and 3rd days of the diet remains constant in each individual throughout the 4th, 5th, and 6th days, whereas it is a universal experienc that even under the most constant conditions urea excretio does not remain absolutely uniform in any individual. Folin[8] for instance found a variation of 5.6 to 8.1 gm. of urea withi a few days in the same individual on his starch-cream diet.

A simple inspection of the arrangement of the subjects in Tabl III in the fore-period as compared with the urea period strongl suggests that we have failed to measure accurately the rate o protein catabolism. Those subjects who have the highest excretion in the fore-period have very often the lowest excretion o administered urea, and *vice versa*. It would seem that we hav overestimated the rate of protein catabolism in some and underestimated in others, an error which introduces a false variabilit in the calculated rates of excretion of administered urea.

It is probable therefore that in reality the rate of excretio of administered urea by the normal kidney has a greater uniformity than that recorded by our figures, since they are influence not only by the possible variability of kidney action, but als by the known variability in each individual from day to day o all those extra-renal factors which are conveniently groupe under the term "protein catabolism."

In order to obtain a conception of the true variability of kidne function in dealing with administered urea, we must compar a group of subjects who took no urea with a group of those wh did. If it were found that there was a greater degree of variability in the urea group, it would be permissible to ascribe th increase in variability to inconstancy in the rate of excretion o administered urea by the kidneys.[9] The difference in the var ability of the two groups would be a measure of the variabilit of kidney function.

We accordingly placed six subjects under the same conditio observed for the urea group recorded in Table III, except tha they took no urea. Any differences between the individu averages of the 2nd and 3rd days and the amounts of urea excreted on the 4th, 5th, and 6th days, represent the extent b which we would have overestimated or underestimated the amounts of "administered" urea excreted, if they had taken any. These differences for each subject and for each period are detailed in Table IV in the same way as for the urea group. When the average of the 2nd and 3rd days is exceeded, the difference is given as plus; when it is less, the difference is minus.

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[8] Folin, O., *U. S. Dept. Agric. Report*, 1911, xciv, 233.

[9] It has been well established, contrary to what might have been expected, that urea has no influence on protein metabolism (see Abderhalde E., *Z. physiol. Chem.*, 1915, xcvi, 1).

In comparing the degree of variability of the groups in Tables III and IV, the average deviation is a much better measure to employ than the simple range of variation, since it takes all the variations into account, and not only the most extreme. The average deviation[10] is the average of all the differences between the individual amounts and the average amount for each group. These differences are added without paying attention to plus or minus signs, and the sum divided by the number of individuals in the group gives the average deviation.

These values are given at the foot of Tables III and IV. It will be noted that there is no marked or constant difference in the variability of the two groups at any period. The most representative period of all is the average of the individual 24 hour excretion of the 4th, 5th, and 6th days. The average deviation for this period in the urea group is 1.40, while it is 1.41 for those who did not take urea.

Since there is no appreciable difference in the degree of variability of urea excretion between those who took urea and those who did not, we may conclude that the variability found in the calculated rate of excretion of administered urea may be accounted for by the variation in the rate of protein catabolism from day to day in the same individual. And although we cannot positively assert that there is no variability in the rate of excretion of administered urea[11] yet any variability which may exist must be of slight extent—so slight that we are not able to demonstrate its existence.

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[10] Yule, G. U., An Introduction to the Theory of Statistics, London, 1912, 144.

[11] Dr. E. S. Kilgore, who suggested the use of the average deviation in comparing the variability of the groups with and without urea, also pointed out that the "probable error" of these average deviations was such that some variability in the rate of excretion of administered urea could not be definitely excluded.

This result appears to us to be of particular interest on account of the widely prevalent conception of the essential variability of normal kidney function under supposedly constant conditions, a view which is derived from the work of Schlayer and others.

The Percentage of Administered Urea Excreted During Successive Periods of the 24 Hours.

After the ingestion of 20 gm. of urea there is an almost instantaneous increase in the urea concentration of the blood which continues until a maximum is attained about 2½ hours later. Thereafter there is some decrease, and there follows throughout the remainder of the 24 hours a succession of fluctuations at concentrations which all remain above the level observed before the urea was taken. The total urea excretion is also immediately increased, but there is no definite rise and fall from a maximum, and in general no close relationship is evident between the urea concentration in the blood and the rate of urea excretion. The rate of excretion is greatest during the first hours, and thereafter decreases and reaches its lowest level during the sleep of the night. This decrease, though in general constant, may show considerable fluctuations during short intervals. These points are illustrated in the following protoco which records observations made on a subject who took 20 gm of urea at 8 a.m. while on the diet.

The rapidity of urea absorption is shown by the considerable increase in blood urea concentration as early as 10 minutes afte the urea entered the stomach. Thereafter the urea was entering the blood more rapidly than it was leaving it through the kidneys and into the tissues, so that for 2½ hours at least the blood concentration was rising. As the rate of tissue absorption and kidney excretion became greater than the rate of entry of urea into the blood from the alimentary tract, the blood urea concentration gradually fell. The subsequent fluctuation in blood urea concentration is not atypical, for we have made similar observations in other cases. It is interesting to note that the sharp rise in blood urea concentration is not duplicated in the curves of urea excretion or of urine concentration. The greatest rate ing the total urea of the fore-period from the

6th day.			
hrs. -8 a.m.	12 hrs. 8 a.m.-8 p.m.		8 h 8 a.m.-
Urea.	Subject.	Urea.	Subject.
gm.		gm.	
24.54	Ai_2	17.66	Ai_2
22.84	S_1	17.51	K_1
22.57	B	16.79	S_1
21.39	P_1	16.37	D
21.21	P_2	16.28	Br_1
21.11	Br_1	15.73	O
20.40	Je	15.68	Br_2
20.28	Boy_1	15.55	W
19.95	O	15.51	M
19.94	D	15.35	Boy_1
19.79	Br_2	15.17	P_1
19.73	M	15.02	Bo_1
19.70	Bo_1	14.95	Je
19.33	W	14.67	We
19.26	Bo_2	14.64	K_3
18.33	C	14.46	K_2
18.22	K_2	14.07	B
17.89	K_3	13.78	S_2
17.80	We	13.76	F
17.53	G	13.65	G
17.27	Boy_2	13.62	Cr
16.95	K_1	13.62	J
16.82	Cr	12.95	Ad
16.61	F	12.81	Add
16.10	S_2	12.78	Ai_1
15.84	J	12.72	
15.18	Ad	12.33	
15.03	Add	11.98	
14.79	Ai_1	10.25	
18.85		14.47	
2.06		1.37	

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21.11	Br$_1$	15.73	O
20.40	Je	15.68	Br$_2$
20.28	Boy$_1$	15.55	W
19.95	O	15.51	M
19.94	D	15.35	Boy$_1$
19.79	Br$_2$	15.17	P$_1$
19.73	M	15.02	Bo$_1$
19.70	Bo$_1$	14.95	Je
19.33	W	14.67	We
19.26	Bo$_2$	14.64	K$_3$
18.33	C	14.46	K$_2$
18.22	K$_2$	14.07	B
17.89	K$_3$	13.78	S$_2$
17.80	We	13.76	F
17.53	G	13.65	G
17.27	Boy$_2$	13.62	Cr
16.95	K$_1$	13.62	J
16.82	Cr	12.95	Ad
16.61	F	12.81	Add
16.10	S$_2$	12.78	Ai$_1$
15.84	J	12.72	
15.18	Ad	12.33	
15.03	Add	11.98	
14.79	Ai$_1$	10.25	
18.85		14.47	
2.06		1.37	

TABLE V.

Time at which blood was collected.	Blood urea concentration per 100 cc.	Hourly urea excretion.	Hourly volume of urine.	Urine urea concentration per 100 cc.	Times between which urine was collected.	Remarks.
	gm.	gm.	cc.	gm.		
7.30 a. m.	0.0300	0.91	143	0.64	7–8 a. m.	Rose from bed at 7 a. m.
8.10 " "	0.0427	1.78	193	0.92	8–9 " "	At 8 a.m. began drinking 360 cc. water with 20 gm. urea. At 8.10 breakfast.
8.20 " "	0.0473					
8.40 " "	0.0517					
9.07 " "	0.0547	2.14	109	1.97	9–10 " "	
9.30 " "	0.0577					
10.30 " "	0.0682	2.17	78	2.78	10–11 " "	
11.30 " "	0.0607	1.89	69	2.75	11 a.m.–12 n.	
12.30 p. m.	0.0514	2.00	79	2.53	12 n–1 p.m.	At 12 n. lunch.
1.30 " "	0.0495	2.06	104	1.99	1–2 p.m.	
		2.92	245	1.20	2–3 " "	
3.30 " "	0.0585	1.71	107	1.59	3–4 " "	
		1.45	67	2.17	4–5 " "	At 4 p.m. afternoon meal.
5.30 " "	0.0495	1.70	71	2.38	5–6 " "	
		1.83	85	2.15	6–7 " "	
		1.68	91	1.84	7–8 " "	
8.30 " "	0.0346	1.20	51	2.35	8–9 " "	At 8 p.m. evening meal.
		1.80	75	2.40	9–10 " "	
		0.76	48	1.58	10 p.m.–7 a.m.	Sleeping from 10.30 p.m.–7 a.m.
8.00 a. m.	0 0540	1.58	107	1.48	7–8 a.m.	Rose from bed at 7 a.m.
		0.99	51	1.95	8–9 " "	

of urea excretion corresponds with the maximum volume of urine, not with the highest blood concentration.

The problems raised in connection with the relationship between the rate of urea excretion and the blood urea concentration will be dealt with in a subsequent communication. We refer to the blood here only to emphasize the fact which has already been demonstrated by Marshall and Davis,[12] that administered urea is not heaped up in the blood, but exists for the greater part in the tissues and is gradually withdrawn from them for excretion by the kidneys. But the relatively high degree of uniformity in the rate of passage of the administered urea through the kidneys is not apparent in the figures of the total urea excretion. These figures show irregular fluctuations from hour to hour, due in the main to the varying rate of formation of urea from food and tissue protein. By subtracting the average total excretion of the 2nd and 3rd days from the excretion of the urea period, as has been done in determining the degree of variability in the rate of excretion of administered urea, we eliminate the differences which exist between different subjects, although individual variations will still prevent more than an approximation being reached. Nevertheless it would seem to be advisable to express the calculated rate of excretion of administered urea during different periods of the 24 hours in terms of a percentage of the 24 hour excretion of administered urea, because such an expression may prove of value as a standard of comparison in detecting functional abnormalities of the kidney.

It has long been known that the amounts of the various constituents of the urine tend under normal conditions to be excreted in larger proportion during the day than during the night; it has also been frequently observed that this relationship may be reversed when the kidneys are diseased, so that the night quantities are greater than those of the day—a condition known clinically as nocturia. The current theory as to the reason for nocturia implies that it arises from an inability on the part of the kidneys to overtake all the extra work thrown on them during the day, so that part of the excretory products are retained to be eliminated during the night. If this explanation be correct, our procedure should provide conditions for the revelation of any latent tendency in this direction, for it involves a marked and sudden call for increased work by the kidneys at the commencement of the day period.

We have therefore worked out for each subject the percentage of the 24 hour excretion of administered urea eliminated during the day and night periods, and also during the first 8 hours of the day, the period throughout which the maximum rate of excretion is found. In Table VI these percentages are given for the individual average excretions of the 4th, 5th, and 6th days.

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[12] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

TABLE VI.

The Percentage of the 24 Hour Excretion of Administered Urea Eliminated During Successive Periods of the 24 Hours.

(Calculated from the averages of the 4th, 5th, and 6th days).

8 hrs. of day. 8 a.m.-4 p.m.		12 hrs. of day. 8 a.m.-8 p.m.		12 hrs. of night. 8 p.m.-8 a.m.	
Subject.	Percentage.	Subject.	Percentage.	Subject.	Percentage.
D	68.4	D	85.7	P_2	34.0
F	65.0	We	81.0	Boy_1	30.2
M	63.7	J	80.4	Br_1	30.6
We	62.5	S_2	80.3	K_1	30.1
Br_2	62.2	Boy_2	80.2	Bo_1	29.3
S_2	60.9	F	79.5	K_2	29.3
J	59.9	B	79.4	Ad	29.0
K_1	58.8	Ai_2	79.0	C	28.7
Bo_1	56.8	P_1	78.5	Add	26.5
Cr	56.4	Br_2	77.4	Ai_1	25.2
Br_1	56.0	M	76.7	Cr	25.2
P_1	55.6	G	75.7	Je	24.8
Je	55.4	S_1	75.4	S_1	24.6
S_1	54.1	Je	75.2	G	24.3
K_2	53.5	Cr	74.8	M	23.3
G	53.5	Ai_1	74.8	Br_2	22.6
Ad	53.5	Add	73.5	P_1	21.5
Add	53.4	C	71.3	Ai_2	21.0
Ai_1	51.8	Ad	71.0	B	20.6
K_2	51.0	K_2	70.7	F	20.5
Boy_1	50.8	Bo_1	70.7	Boy_2	19.8
		K_1	69.9	S_2	19.7
		Br_1	69.4	J	19.6
		Boy_1	67.8	We	19.0
		P_2	66.0	D	14.3
Average..	57.1		75.5		24.5

In every case more than one-half of the 24 hour excretion of administered urea appears during the first 8 hours, and there is not more than about one-third at most excreted during the 12 hours of night. The average day excretion is more than three times greater than that of the night.

The existence of a condition described as "kidney fatigue" has been hypothecated by some observers[13] to explain certain experimental results. Whether this view is justified or not, we certainly have no evidence of any such condition in these experiments, although the addition of a heavy load on the work of the kidneys for 3 successive days might be expected to elicit such a state. On the contrary we find that the average percentage excreted during the first 8 hours after urea administration increases slightly, from 54.9 per cent on the 4th day, to 58.3 per cent on the 5th day, to 59.3 per cent on the 6th day.

Urea taken by mouth by virtue of its extreme diffusibility is quickly and evenly distributed throughout all the tissues of the body. As the urea content of the blood is lowered by the secretory activity of the kidneys, the urea is gradually returned from the tissues to the blood, and is excreted. The constancy in the rate of excretion of administered urea shows that there must be a relationship at any one period of the 24 hours between this rate of excretion and the amount of the administered urea still remaining in the body. The exact range of variation in this relationship will of course not be exactly determinable because of the individual variations in protein catabolism, but the averages for the different periods should give a close approximation This relationship, expressed in the form of the percentages of the administered urea within the body which were excreted at successive 4 hour intervals during the day, is given in Table VII

There seems to be a fairly constant relation between the rate of excretion and the amount of administered urea in the body The lowest percentages are found during the first 4 hours of each day. This might have been ascribed to the fact that the 20 gm. of administered urea require some time to be absorbed But this is not a valid explanation, for we know that at the very commencement of this period the blood urea concentration

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[13] D'Amato, L., and Fagella, V., *Z. klin. Med.*, 1911, lxxii, 474. Schlayer, C., *Verhandl. kong. inn. Med.*, 1912, 501. Mosenthal, H., and Schlayer, C., *Deutsch. Arch. klin. Med.*, 1913, cxl, 217. Lindemann, *Ergebn. Physiol.*, 1914, xix, 652.

TABLE VII.

The Relationship between the Average Amounts of Administered Urea Excreted During Successive 4 Hour Intervals after the Ingestion of 20 Gm. of Urea, and the Average Amounts of Administered Urea within the Body.

Day.	Period.	Administered urea excreted in 4 hour periods.	Administered urea in body at commencement of each period.	Percentage of the administered urea in the body which was excreted every 4 hours.
4th	8 a.m.–12 n.	4.82	20.00	24
	12 n.–4 p.m.	4.37	15.18	29
	4–8 p.m.	3.23	10.81	30
5th	8 a.m.–12 n.	5.64	23.02	24
	12 n.–4 p.m.	5.37	17.38	31
	4–8 p.m.	3.38	12.01	28
6th	8 a.m.–12 n.	6.01	24.22	25
	12 n.–4 p.m.	5.23	18.21	29
	4–8 p.m.	3.24	12.98	25

rises rapidly to a level above that existing during the remainder of the day, so that the mean blood urea concentration is higher for this period than for any other. One would therefore expect that the largest instead of the lowest proportion of the administered urea in the body would have been eliminated. This is a good example of the fact that there are conditions under which there is not always a direct relationship between the concentration of urea in the blood and the rate of urea excretion.

The Rate of Excretion of Administered Urea after the Ingestion of 40 Gm. of Urea.

After the above data on the rate of urea excretion after the ingestion of 20 gm. of urea had been accumulated, we proceeded to determine the effect of the administration of double that amount of urea.

Nine subjects took 40 gm. of urea under the same conditions as before, except that the subjects K_4, Bo_3, Br, and Ca took the urea dissolved in 720 cc. of water instead of 360 cc. Unfortunately 4 hour collections were not made during the fore-period, so that we can only give the rate of excretion of administered urea for 24 hour and 12 hour periods. The results are given in Table VIII.

TABLE VIII.

Total urea excreted (fore-period).				Administered urea excreted after the ingestion of 40 gm. of urea.															
Average of 2nd and 3rd days.				4th day.				5th day.				6th day.				Average of 4th, 5th, and 6th days.			
24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m. 8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.		24 hrs. 8 a.m.-8 a.m.		12 hrs. 8 a.m.-8 p.m.	
Sub-ject.	Urea.	Sub-ject.	Urea.	Sub-ject.	Urea.	Sub-ject.	Urea.	Sub-ject.	Urea.	Sub-ject.	Urea	Sub-ject.	Urea.	Sub-ject.	Urea.	Sub-ject.	Urea.	Sub-ject.	Urea.
	gm.		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*		*gm.*
Bo$_3$	19.18	T$_2$	11.03	Ca	38 16	Pr	29.42	Lo	42.74	St$_2$	32.40	Pr	47.10	Lo	32.85	Pr	40.66	Pr	30.60
T$_2$	18.83	Bo$_3$	10.00	Pr	37.64	Ca	28.74	St$_2$	41.79	Lo	31.65	Lo	45.38	St$_2$	32.52	Lo	40.44	Lo	29.87
K$_4$	17.25	Lo	9.75	Bo$_3$	36.62	Bo$_3$	28.28	T$_2$	40.33	Pr	31.44	T$_2$	42.97	T$_2$	31.81	T$_2$	39.21	T$_2$	29.57
Ca	17.04	Ca	9.66	Ba	36.05	Ba	28.07	Ba	38.33	T$_2$	30.73	St$_2$	41.43	Sh$_2$	31.16	Ca	39.08	Ca	29.22
St$_2$	15.57	K$_4$	9.21	Sh$_2$	35.15	Sh$_2$	27.68	Ca	37.44	Ba	29.39	Sh$_2$	40.79	Pr	30.96	Ba	38.01	Ba	28.71
Pr	15.42	Ba	8.65	T$_2$	34.34	T$_2$	26.17	Sh$_2$	37.31	Ca	28.26	Ca	40.66	Bo$_3$	30.74	Sh$_2$	37.75	Sh$_2$	28.48
Ba	14.59	St$_2$	8.58	K$_4$	33.51	Lo	25.13	Pr	37.26	K$_4$	28.03	K$_4$	39.63	Ca	30.66	K$_4$	36.36	Bo$_3$	28.38
Lo	14.50	Pr	8.52	Lo	33.22	K$_4$	24.39	K$_4$	35.95	Sh$_2$	26.60	Ba	39.63	K$_4$	30.63	Bo$_3$	35.80	K$_4$	27.68
Sh$_2$	13.57	Sh$_2$	7.12					Bo$_3$	32.90	Bo$_3$	26.12	Bo$_3$	37.88	Ba	28.89				
Aver-age..	16.22		9.17		35.59		27.21		38.23		29.40		41.72		31.14		38.41		29.06
Average deviation......					1.53		1.50		2.28		1.91		2.29		0.84		1.43		0.76

The average deviations show that the rate of excretion of adninistered urea is quite as constant after 40 as after 20 gm.

The average percentage of the 24 hour excretion of administered rea eliminated during the day and night periods was 75.6 per ent for the day time and 24.4 per cent for the night, as compared ith 75.5 and 24.5 per cent after the ingestion of 20 gm. of rea. The ranges of variation were from 78 to 74 per cent for he day, and from 26 to 22 per cent for the night.

The relationship between the rate of excretion of administered rea and the amount of administered urea within the body is lso substantially the same. After 40 gm. of urea the percentage f the administered urea within the body which was excreted luring the 12 hours of the day was 64 per cent, whereas after 0 gm. of urea it was 61 per cent.

At the completion of the urea period an average of 4.46 gm. f the administered urea remained within the body when 40 gm. f urea were taken. The average amount after 20 gm. was 4.96 m. The retention of this urea is not to be ascribed to an inabilty on the part of the kidneys to excrete it. It is retained beause it has never been brought to the kidneys for excretion.

It would be useless to attempt by giving larger doses of urea o reach any absolute limit to the capacity of the normal kidney n excreting urea since no such limit exists for any dose of urea hat it is possible to give. As long as sufficient water is available the rate of excretion will increase with every increase in he amount of urea ingested.

CONCLUSIONS.

1. In thirty-nine experiments on young healthy adults no evience of variability in kidney action could be obtained in the ork of excreting preformed urea added to a constant diet. Such ariation as was found in the amounts of urea excreted could be ully accounted for as arising from extra-renal factors. It is oncluded therefore that the normal kidney under constant onditions is characterized by the possession of a high degree of onstancy of function.

2. The rate of excretion of the administered urea during sucessive periods of the 24 hours showed that the repetition of

large doses of urea did not elicit the condition which has been described as "kidney fatigue."

3. A fraction of the urea which was administered remained in the body after 24 hours. This urea was not retained because of any failure on the part of the kidneys to eliminate it, for the amount retained was no larger when 40 than when 20 gm. of urea were taken.

THE VOLUME OF URINE IN YOUNG HEALTHY ADULTS ON A CONSTANT DIET.

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In the course of an investigation into the conditions controlling the rate of urea excretion, we have accumulated data in regard to the volume of urine of subjects on a constant water intake, which are of themselves of interest as illustrating the wide range of variation of this factor, and may further prove of value in defining the limits beyond which a given volume of urine may properly be regarded as abnormally large or small.

The diet has been detailed in the previous paper on the rate of urea excretion. The total water content of the food and fluids was 1,710 cc. This was increased to 2,070 cc. on the last 3 days of the diet by the 360 cc. of water in which the urea taken was dissolved. A slight inequality in water intake was introduced in those few cases who did not always take all of the nitrogen-free cornstarch which was provided..

Table I gives the averages and limits of variation in the volume of urine in those twenty subjects who took 20 gm. of urea under the above conditions.

It is evident from these figures that the volume of urine in any individual for any one day or part of a day is an extremely variable quantity, in spite of constant water intake. But when the volumes of the 4th, 5th, and 6th days in each individual are averaged, the range of variation is greatly lessened. These averages are given in order of ascending magnitude in Table II.

We hope to find the above figures useful in the recognition of true polyuria or oliguria.

When the kidneys are diseased, the volume of urine passed at night is often larger than the day urine. But we have found that this condition may be present in subjects whose kidneys are perfectly normal. When, however, the percentages of the total 24 hour urine excreted at night on the 4th, 5th, and 6th days are averaged for each individual, we find that in all of them the night excretion is less than the day. Table III shows the average and limits of variation in the average percentage distribution of urine volume for 3 days.

TABLE I.

Average and Limits of Variation in Volume of Urine (Cc.).

Period.	1st day.			2nd day.			3rd day.		
		Limits of variation.			Limits of variation.			Limits of variation.	
	Average.	Highest.	Lowest.	Average.	Highest.	Lowest.	Average.	Highest.	Lowest.
24 hrs., 8 a.m.-8 a.m.	1,058	2,215	609	1,059	2,023	727	975	1,341	587
12 hrs., 8 a.m.-8 p.m.	642	1,405	381	646	1,272	366	617	862	395
8 hrs., 8 a.m.-4 p.m.	458	1,141	281	413	581	225	417	646	284
	4th day.			5th day.			6th day.		
24 hrs. 8 a.m.-8 a.m.	1,406	2,323	951	1,354	1,674	1,003	1,322	1,665	903
12 hrs., 8 a.m.-8 p.m.	902	1,681	605	913	1,248	732	893	1,269	615
8 hrs., 8 a.m.-4 p.m.	643	1,149	422	661	925	446	645	1,035	477

There is not less than 38 per cent of the 24 hour volume excreted during the first 8 hours of the day, and not less than 5 per cent in the first 12. Under these conditions also we nev find more than 47 per cent eliminated during the night.

We may therefore be reasonably certain of the presence o true nocturia, meaning an abnormally high proportion of ni urine, when we find in any individual who takes the diet that per cent or more of the average 24 hour excretion of the la days is eliminated at night.

TABLE II.

The Average Volume of Urine of the 4th, 5th, and 6th Days.

No.	8 hrs. (8 a.m.—4 p.m.)		12 hrs. (8 a.m.—8 p.m.)		24 hrs. (8 a.m.—8 a.m.)	
	Subject.	Volume.	Subject.	Volume.	Subject.	Volume.
		cc.		*cc.*		*cc.*
1	Br$_1$	501	A$_1$	684	A$_1$	1,013
2	A$_1$	519	Br$_1$	709	Br$_1$	1,022
3	Ad	519	Ad	713	Ad	1,097
4	Bo$_1$	527	Bo$_1$	740	S$_1$	1,202
5	G	528	D	813	Bo$_1$	1,213
6	D	603	G	819	F	1,234
7	J	604	K$_2$	833	We	1,253
8	P	613	F	868	D	1,257
9	F	626	P	879	P	1,297
10	K$_2$	626	J	901	J	1,345
11	Cr	691	K$_1$	901	G	1,378
12	K$_1$	700	We	924	Cr	1,393
13	We	707	Boy$_1$	935	Boy$_1$	1,399
14	B	708	B	964	S$_2$	1,441
15	S$_1$	724	K$_3$	968	M	1 441
16	S$_2$	734	Cr	1,011	B	1,467
17	K$_3$	734	S$_2$	1,017	K$_3$	1,489
18	Boy$_1$	765	S$_1$	1,042	K$_2$	1,526
19	M	773	M	1,063	Je	1,707
20	Je	788	Je	1,195	K$_1$	1,712
Average.		650		903		1,361

TABLE III.

Average and Limits of Variation in the Average Percentages of Urine Volume Excreted During Different Periods of the 24 Hours on the 4th, 5th, and 6th Days.

Period.	Average.	Highest.	Lowest.
	per cent	*per cent*	*per cent*
8 hrs. of day, 8 a.m.–4 p.m.	48	56	38
12 " " " 8 a.m.–8 p.m.	66	74	53
12 " " night, 8 p.m.–8 a.m.	34	47	26

In order to determine whether a moderate increase in the volume of urine led to any increase in the rate of excretion of administered urea, the experiment was repeated in four subjects under the same conditions except that 250 cc. of water were taken with each of the four meals, in all 1,000 cc. of additional water for each 24 hour period. Table IV gives the average amounts of administered urea excreted by these four individuals with and without extra water.

TABLE IV.

No Extra Water.

Period.	Average amounts of administered urea excreted.				Volume of urine.
	4th day.	5th day.	6th day.	Average of 4th, 5th, and 6th days.	Average of 4th, 5th, and 6th days.
					cc.
24 hrs., 8 a.m.–8 a.m.	15.3	20.0	19.9	18.4	1,128
12 " 8 a.m.–8 p.m.*	11.3	15.7	14.1	13.7	752
8 " 8 a.m.–4 p.m.**	9.3	11.5	10.6	10.5	545
1,000 Cc. Extra Water Per Day.					
24 hrs., 8 a.m.–8 a.m.	17.0	19.4	20.4	18.9	2,350
12 " 8 a.m.–8 p.m.*	12.2	13.7	16.3	14.1	1,671
8 " 8 a.m.–4 p.m.**	10.2	12.2	13.2	11.9	1,277

*Average of three cases only.
** " " two " "

While the figures for the 4th and 6th days suggest a slight acceleration of the rate of excretion, yet the averages for the 3 days are almost identical. These differences are well within the limits of error, and it therefore cannot be said that there is any demonstrable increase in the rate of excretion of administered urea when 1,000 cc. of water a day are added to the diet. We do not conclude that the volume of urine never has any effect on the rate of urea excretion. On the contrary, we have evidence which will be given later that under certain well defined conditions the volume of urine is a very important factor in determining the rate of excretion. But under the above conditions doubling the volume of urine has no appreciable influence.

It may be worth noting that there is no indication that the extra water altered the level of protein catabolism. The average total excretion of urea for the whole period of 6 days was 152 gm.

when the total urine volume was 6,484 cc., and 149 gm. when the volume was 12,827 cc.

Although the rate of urea excretion is not demonstrably increased by an increase in urine volume, an increase in the rate of urea excretion induced by the ingestion of urea is accompanied by an increased urine volume. The average volumes of urine excreted on the 4th, 5th, and 6th days of the diet by three groups of individuals who took different amounts of urea are compared in Table V. The first group consists of six individuals who were given 360 cc. of water but no urea, the second comprises twenty who took 20 gm. of urea in 360 cc. of water, and the third group is compiled from five individuals who were given 40 gm. of urea in 360 cc. of water.

TABLE V.

The Average Volume of Urine of the 4th, 5th, and 6th Days in Groups of Individuals Who Took No Urea, 20 Gm. of Urea, or 40 Gm. of Urea.

Period.	No urea.	20 gm. of urea.	40 gm. of urea.
	cc.	*cc.*	*cc.*
24 hrs., 8 a.m.–8 a.m.	1,051	1,361	1,446
12 " 8 a.m.–8 p.m.	707	903	988
8 " 8 a.m.–4 p.m.	547	650	735
4 " 8 a.m.–12 noon	245	337	398

In spite of the constancy of the water intake the average volume of urine is considerably increased in those who took urea.

CONCLUSIONS.

1. The volume of urine in normal individuals on a constant diet with the same water intake is extremely variable for any single day or part of a day.
2. The average volume of the last 3 days of the diet when the water intake was 2,070 cc. varied in twenty individuals from 1,013 to 1,712 cc. for a 24 hour period, from 684 to 1,195 cc. for the first 12 hours of the day, and from 501 to 788 cc. for the first 8 hours of the day.
3. The percentage of the 24 hour volume excreted during the 12 hours of the night did not exceed 47 per cent in any subject, when the volumes for the last 3 days of the diet were averaged.

4. An increase of 1,000 cc. a day in the water intake more than doubled the volume of urine but did not appreciably increase the rate of urea excretion.

5. An increase in the rate of urea excretion induced by the ingestion of urea, the water intake remaining constant, was accompanied by a considerable increase in the volume of urine.

OBSERVATIONS ON THE CHANGES IN THE CHOLESTEROL CONTENT OF THE BLOOD OF GOATS, FOLLOWING CHOLESTEROL FEEDING ALONE, ROENTGEN TREATMENT ALONE, AND CHOLESTEROL FEEDING COMBINED WITH ROENTGEN TREATMENT AND SUBSEQUENT CASTRATION.*

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(Received for publication, July 4, 1916.)

The importance of cholesterol metabolism has been established by the work of Weltmann (1), Bacmeister and Havers (2), Aschoff (3), McNee 4), Wacker (5), Sternberg (6), Rothschild (7), and others; that its study s becoming a subject of general interest may be seen from the number of observations that have been made within the last 2 years.

While the main object of the earlier work done on cholesterol was the xperimental reproduction of the arteriosclerotic lesions (Stuckey (8), Vesselkin (9), Chatalow (10), Anitschkow (11), and lately McMeans 12)), this work proved incidentally that a great number of organs are ffected by a disturbance of the cholesterol balance. Subsequent inestigations have shown that the organs principally concerned with the egulation of the cholesterol metabolism are the adrenals, the liver, the enital glands, and the intestines (Rothschild (7), Weltmann and Biach 13), Anitschkow and Chatalow (14), Albrecht and Weltmann (15), Stewart (16), Hueck (17), Gardner and Lander (18), Sternberg (6), Lowenthal, 19), McMeans (12), McNee (4)). That cholesterol is also stored in the ody fat has been demonstrated by Rothschild (20) and Wacker (5). It as further been shown that the cholesterol content of the blood is changed y physiologic as well as by pathologic conditions; that pregnancy and actation, diseases of the liver, and malignant growths increase cholesterol alue, but that the influence of bacterial infection depends on the acute or hronic character of the process; Weltmann (1) found that in tuberculosis, or instance, the cholesterol content of the blood was lowered as the disease progressed.

The effect of cholesterol on cell proliferation has been made a subject of recent investigations: Robertson and Burnett (21) reported that a growth of transplanted carcinoma in white rats (Flexner-Jobling type) was accelerated by the intravenous injections of cholesterol emulsion; Browder (22) states that the rate of cell division of paramecia is markedly increased by the addition of small amounts of cholesterol to the culture medium; McMeans (12) observed that proliferation of the arterioles of the lungs and the kidneys occurred in cholesterol-fed rabbits. The bearing of these experiments on the problem of malignant growth is obvious.

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* Read by title before the American Association of Pathologists and Bacteriologists, Washington, D. C., May 9–10, 1916.

The foregoing brief review shows that a great number of factors influence cholesterol metabolism; *viz.*, the relative adequacy of some four or five organs taking part in the regulation of the metabolic balance and physiologic as well as pathologic conditions. There may be other factors whose importance has not yet been ascertained, and it will therefore be easily understood that the interpretation of changes in the cholesterol value of the blood is fraught with difficulties. At present only deductions based on unusually high, or unusually low, on markedly progressive, or markedly recurrent cholesterol values can be expected to give any definite clue concerning the actual relation between a disturbance of the cholesterol balance and the associated pathologic symptoms.

The object of my experimental work is to produce malignant proliferation in animals by disturbing the cholesterol balance. For this purpose cholesterol feeding has been used. In some cases Roentgen treatment has been employed in addition, to break down the "lymphoid defence" of the animal (Murphy and Morton (23)). Cholesterol tests of the blood were made, not as an end in themselves, but as a means by which the progress of the metabolic disturbance might be gauged. A few observations made during the course of the experiment seem to furnish data concerning some of the factors that influence the cholesterol content of the blood. These observations I wish to present here although the experiment under discussion is not yet completed.

Rothschild (7) in his studies on the cholesterol content of the blood of rabbits found that normal animals appear to have an "individual cholesterol standard" to which they adhere with only slight variations. Bloor (24) has observed that in dogs the process of digestion and the chemical constituents of certain types of food do not influence the cholesterol percentage to any considerable extent. In the main, this may also be applied to goats (Table I, Fig. 1).

TABLE I.

Cholesterol Values in the Blood of Normal Goats. Tests Made Every 2 Hours (8 a.m. to 6 p.m.). Results Expressed in Per Cent.

Time.	Readings.*	Highest value.
	Goat 21 (male; received cholesterol only).	
8 a.m.	0.216 - - 0.216 - - 0 272** --- (0.216)†	0.272
0 "	0.186 - - 0.216 - - 0.254 - - 0.254 - - 0.266** ----- (0.230)	0.266
2 m.	0.242 - - 0.242 - - 0.262 - - 0.262 - - 0.266** ------ (0.216)	0.266
2 p.m.	0.254 - - 0.254 - - 0.266** ------ (0.224)	0.266
4 "	0.216 - - 0.216 - - 0.242 - - 0.242 - - 0.272** ----- (0.242)	0.272
6 "	0.210 - - 0.210 - - 0.242 - - 0.242 - - 0.260** ---- (0.220)	0.260
	Goat 10 (male; normal control).	
8 a.m.	0.158 - - 0.158 - - 0.180 - - 0.180 - - 0.202** ------ (0.168)	0.202
0 "	0.146 - - 0.146 - - 0.186** ------- (0.158)	0.186
2 m.	0.158 - - 0.158 - - 0.186** -------- (0.158)	0.186
2 p.m.	0.168 - - 0.168 - - 0.190 - - 0.190 - - 0.202** ------ (0.158)	0.202
4 "	0.186 - - 0.186 - - 0.202** ------ (0.168)	0.202
6 " ‡	0.130 - - 0.130 - - 0.186** -------(0.146)	0.186

*The readings made at each 2 hour period were repeated at intervals of 2 minutes.

** Value remained constant for four or more readings.

† The small dashes represent approximately the number of minutes hat elapsed between the last constant reading and the first reading lower. Note that this interval was longer for Goat 10 than for Goat 21.

‡ The slightly lower values at this time may have been due to the fact hat a few clots had formed.

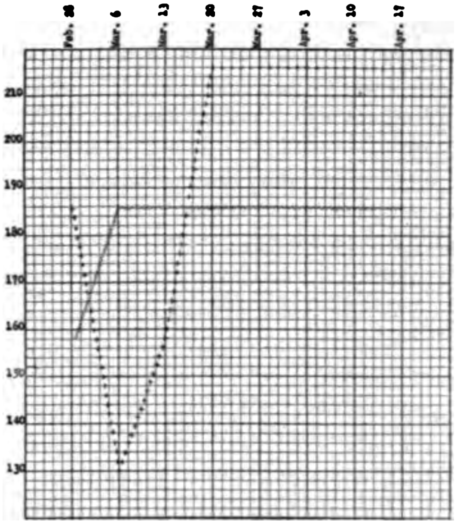

FIG. 1. Cholesterol values in the blood of two normal goats; Goat 10, male, ∿∿∿∿∿; Goat 35, female, +++. Note that the values for Goat 10 remained constant for 7 weeks and those for Goat 35 remained constant for 5 weeks.

Fig. 1 shows the cholesterol values found in the blood of two normal goats, one male (G10) and one female (G35), during a period of 8 weeks (weekly tests). Whereas the values of the male animal remained unchanged for 7 consecutive weeks, a drop occurred in the cholesterol value of the female between March 6 and 13, the animal being in heat at the time. The fact that a lower cholesterol value was observed while the animal was in heat indicates that the cholesterol content of the blood is influenced by reproductive activity, an observation to be discussed at greater length in connection with the period following castration.

Table I gives the cholesterol values found by tests made every 2 hours from 8 a.m. to 6 p.m. The effect of the various stages of digestion even in a ruminating animal should be clearly demonstrable by an experiment extending over a period of 10 hours. Whether the slight variations that occurred are really due to the digestive process is a difficult matter to decide, since a goat ruminates and digests without interruption so that even if food were kept out of its reach for some time, the digestive factor could not be entirely eliminated. (Starvation increases the cholesterol content, as Rothschild has ascertained. However, it would seem practically impossible to regulate the food supply for a ruminating animal so that digestion would cease without risking the occurrence of slight symptoms of starvation.) Whether the fluctuations of the cholesterol content (Table I) are due to the process of digestion or to a greater amount of muscular activity on the part of the animal, they are in themselves so slight that they can hardly be considered significant.

Method of Investigation.—In my experiment, the cholesterol content of the blood of six goats was determined every week in the course of 7 months. For obvious reasons this part of the experiment is divided into three periods: (1) Observation and cholesterol feeding alone (Fig. 2). (2) Continued cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment (Figs. 3a, 3b, and 3c). (3) Following castration, other experimental factors unchanged (Figs. 5a, 5b, and 5c).

First Period.—During the first 5 weeks all the goats were kept under good laboratory conditions and were not subjected to any experiment except that 3 cc. of blood were taken from the jugular vein by means of a hypodermic syringe every week in order to determine the average cholesterol value of the blood of each animal. At the end of 3 weeks all the animals developed symptoms of mange. The disease remained confined to small areas on the ears or nose and apparently cleared up in a fortnight. The changes occurring in the cholesterol value from Oct. 7 to Nov. 1 may be partly accounted for by this infection, and partly by the fact that the goats, having been on free range, had not yet become acclimated to laboratory conditions and stable food. On Nov. 1 two of the animals (G19 and G21) were fed daily 0.30 gm. of cholesterol in capsules, that the effect of this substance on the cholesterol content of the blood might be studied. The extraordinary digestive powers of the goat which cause this animal to thrive on the most indigestible articles seemed to justify the apprehension that the cholesterol might be eliminated without any apparent effect. However, as may be noted from Fig. 2, anxiety on this point proved without foundation, for whereas two of the controls (G16 and G26) did not make up the deficit in their cholesterol value, and the other two (G17 and G20) exceeded their original percentage by a small margin only (0.006–0.018), the two cholesterol-fed animals (G19 and G21) gained 0.90 per cent; that is, the cholesterol content of the blood increased by 0.090 and 0.062 mg. respectively, as compared with the original values. Therefore, from Fig. 2, the following conclusions may be drawn.

1. The cholesterol content can be increased in the blood of goats by cholesterol feeding.

2. Parasitic infection (mange) apparently decreases the cholesterol value of the blood. This deduction has been justified by later observations during a recurrence of mange after the animals had become thoroughly acclimated to laboratory conditions and were steadily putting on weight. The fact may perhaps be explained as the result of an effort on the part of the organism to combat the infection.

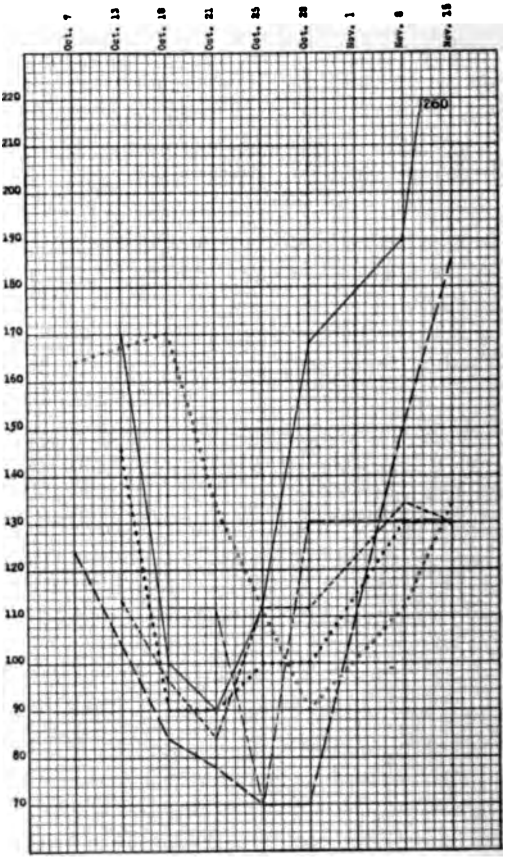

FIG. 2. Cholesterol values for Period I. Goat 16, ° ° °; Goat 17, — - —; Goat 19, ———; Goat 20, – – –; Goat 21, —— —— ——; Goat 26, ... Note the drop in the values for all of the goats during infection (mange and the increase in the values for Goats 19 and 21 on cholesterol feeding.

Second Period.—During the second period observations were made on the effect of cholesterol feeding alone, of Roentgen treatment alone, and of Roentgen treatment combined with cholesterol feeding, the six goats being divided into three corresponding groups; *viz.*, G19 and G21 (Group A) continued to get 0.30 gm. of cholesterol daily in capsules; G16 and G26 (Group B) diffuse Roentgen treatment (diaphragm wide open) consisting of a 12.5 milliampere per minute dose daily for 7 days (2.5 milliamperes at a distance of 24 inches from the tube, exposure 5 minutes) followed by 7 days' rest; G17 and G20 (Group C) were given the same Roentgen treatment but received in addition 0.30 gm. of cholesterol daily without interruption (Figs. 3a, 3b, and 3c).

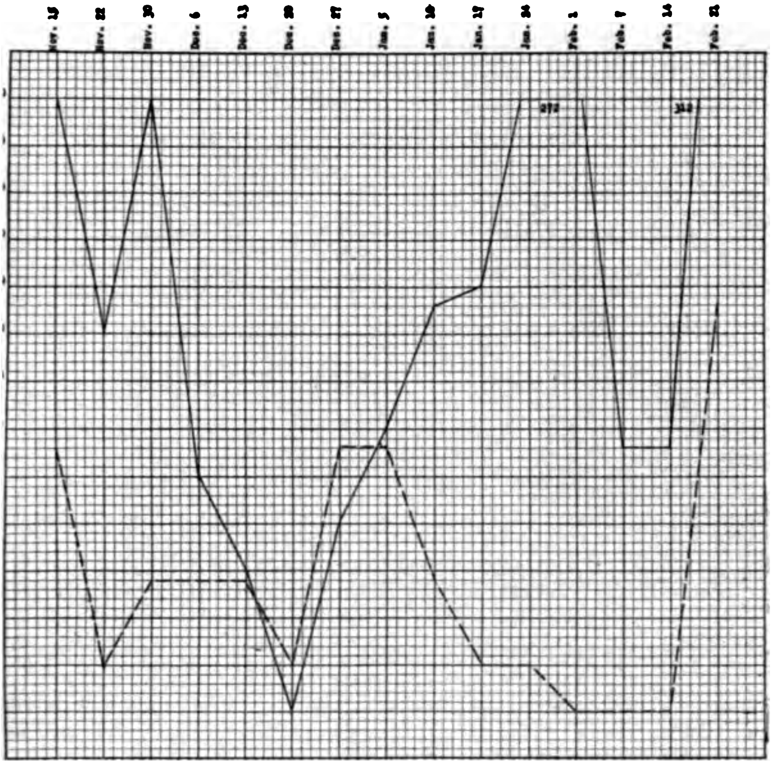

Fig. 3a. Cholesterol values for Group A, Period II. The effect of cholesterol feeding and mange. Goat 19, ———; Goat 21, — — —.

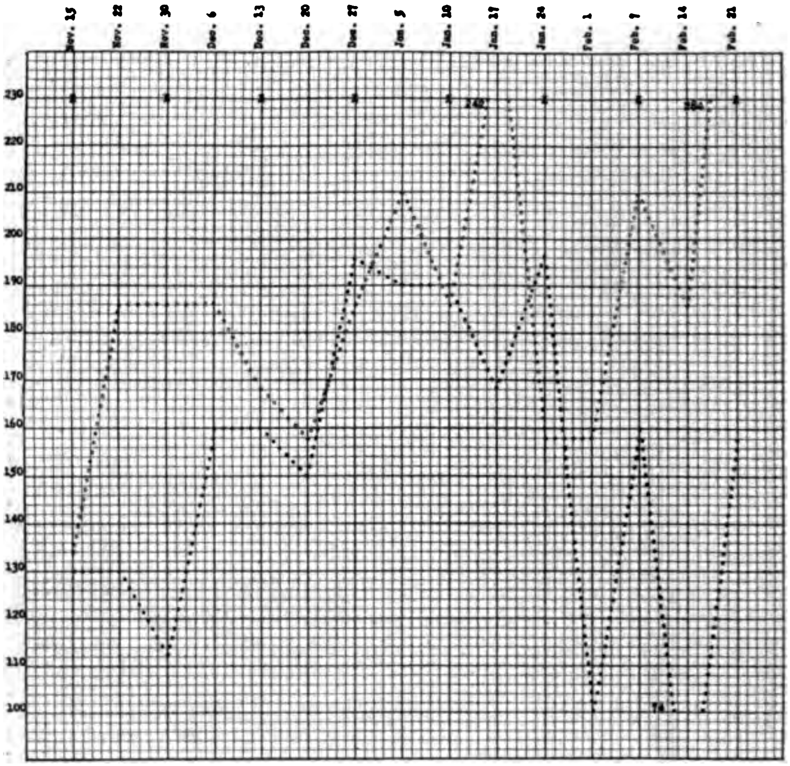

FIG. 3 b. Cholesterol values for Group B, Period II. The effect of Roentgen ray treatment and mange. Goat 16, ° ° °; Goat 26 ...... X indicates Roentgen ray treatment.

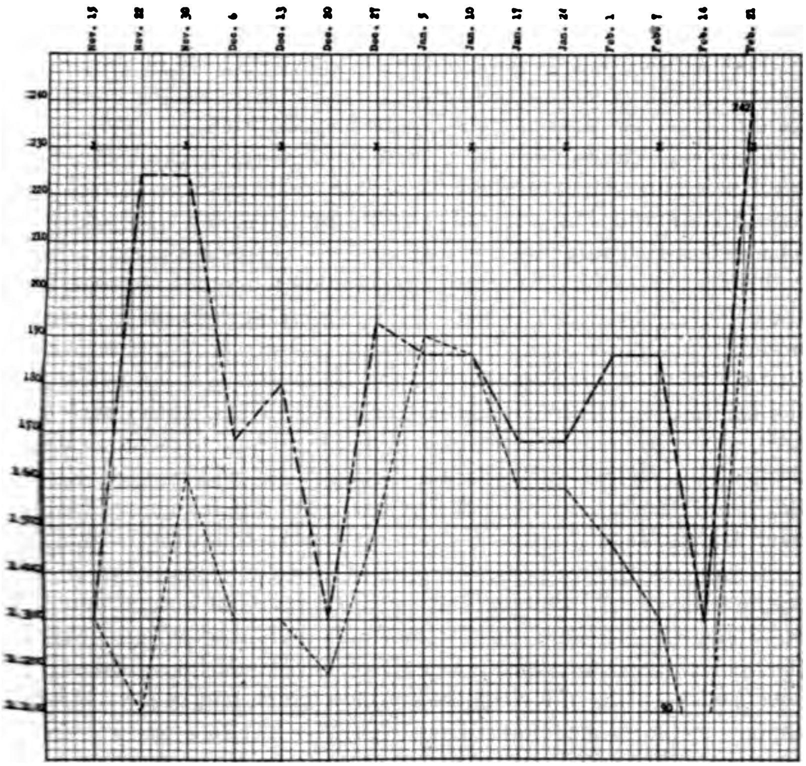

Fig. 3 c. Cholesterol values for Group C, Period II. The effect of Roentgen ray treatment and cholesterol feeding combined (in the values for Goat 17, the additional influence of pregnancy). Goat 17, —— - ——; Goat 28, — — —. X indicates Roentgen ray treatment.

The interpretation of the results obtained during this period is extremely difficult owing to the number of intercurrent factors, the effect of which on the cholesterol content of the blood can be merely guessed, since at present data for comparison are not available and can be obtained only by further investigations. On the other hand, the conclusion which seemed warranted by my observations during the first period, *viz.*, the increase of the cholesterol value by cholesterol feeding and the decrease of the cholesterol value by mange, appear to be corroborated.

Thus, G19 (Group A), which already had the highest cholesterol values during the previous period, will again be found to have higher values than any of the other animals, and G21, which, although it had responded to the cholesterol feeding previously, had done so to a lesser degree, again shows a general increase in the cholesterol content of its blood, less marked than the increase found in the other animal of the same group. Closer examination of the curves in Figs. 3a, 3b, and 3c reveals the influence of the intercurrent factors referred to above. Thus, in the case of G19, a severe recurrence of mange on the shoulder explains the drop of the cholesterol value on Nov. 22. It will be seen that the value went up with the disappearance of the mange. Moreover, the cholesterol dropped again during the following weeks, a decrease that may be partly accounted for by another recurrence of the mange. All the goats except G16 and G20 were affected. In the case of G19, another factor which undoubtedly influenced the cholesterol content from Dec. 13 onward must be considered; *viz.*, the formation of a cyst-like swelling which had become large enough to be noticeable on Dec. 30. This swelling was located near the right nostril, extended over the upper lip, and measured about 2 cm. in diameter. Slight fluctuation was present at first, suggesting that it might be an abscess. The fluctuation then disappeared, and the swelling assumed a tumor-like aspect. To ascertain the exact nature of the tumor, it was lanced on Jan. 17, and found to contain pus. A small quantity (1.2 gm.) of the pus was taken for chemical analysis, and the remainder left to be reabsorbed by the animal. A test of the pus for cholesterol showed the cholesterol value to be ten times that of the blood and seemed to warrant the assumption that the abscess was a "deposit" for the increasing surplus cholesterol in the blood. This supposition appeared to be corroborated by the increase of the cholesterol value in the blood during the following weeks, when the remainder of the abscess was being slowly reabsorbed. Several tests made with pus from patients did not give any higher cholesterol values than are usually found in the blood under conditions of slight inflammation. The fluctuations in the cholesterol value of the blood of G21 would appear to be due to the appearance and disappearance of mange.

For the changes in the cholesterol values observed in all of the animals between Feb. 1 and 21, no apparent reason can be found unless extreme changes in the temperature (days on which the thermometer registered 25–40° below zero, alternating with days of warm spring-like weather) are to be taken into account.[1] It is quite conceivable that similar extremes of heat and cold cannot be without effect on general metabolism.

The result of the Roentgen treatment (Group B) is not sufficiently striking to permit of any definite conclusions. The fact that a slight drop in the cholesterol value occurs in several instances toward the end of the second period after a week of Roentgen treatment (Jan. 24 to Feb. 21, G16, G17, G20, and G26), and is followed by a slight increase after a week of rest, suggests the possibility that the Roentgen ray may have a depressing effect on the cholesterol value in the blood. Similar changes were found to occur during the third period. The present data are still too meager to furnish any conclusive evidence although they appear to be in accordance with Soper's (25) observations on the effect of mesothorium rays on the cholesterol value in the blood of rabbits. Soper reports a decrease of the cholesterol content in his animals after mesothorium treatment, but considers it too slight to be significant since it was not corroborated by histologic changes in the spleen. The short duration of his experiment (9 to 11 days) and the fact that several organs play a part in the regulation of cholesterol metabolism might explain his results. If, however, the reduction of the cholesterol content by Roentgen rays should become more marked in the course of my experiments or be confirmed by independent studies, it might furnish valuable information concerning the beneficial effect of the Roentgen rays on malignant conditions, since the stimulating effect of cholesterol on cell proliferation demonstrated experimentally, and the high cholesterol values found in the blood of a number of cancer patients, seem to indicate that there may be a close connection between disturbances of cholesterol metabolism and malignant growth.

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[1] Though the animals were kept in stalls, warmed by the central heating system, there was an outside run in which they spent a great part of the day, apparently by preference.

The possible effect of extreme and sudden changes of temperature on the cholesterol content of the blood has been mentioned in the discussion of Group A.

In Group B, the influence of mange on the cholesterol values is but faintly indicated. G26 alone had a slight recurrence, and it is difficult to decide whether the drop from 0.160 to 0.150 per cent (Dec. 13 to 20) is to be attributed to the disease or to the Roentgen treatment. On Jan. 10 G16 developed about half a dozen small abscesses in the inguinal region, none of which were larger than a pea. These abscesses cleared up without treatment and on Feb. 7 had completely disappeared. As the postules were too small to permit the taking of any pus for chemical analysis, it is impossible to say whether their reabsorption could have caused the changes found in the cholesterol value on Jan. 10 and 17, or were in any way comparable to the abscess on the nose of G19.

By combining Roentgen treatment with cholesterol feeding (Group C) I hoped to ascertain which of the two forms of treatment exerted the greater influence on the cholesterol content of the blood. The time allowed for this part of the experiment, 4 months, seemed amply sufficient to warrant definite results. However, the cholesterol values registered do not furnish conclusive data, although they suggest that Roentgen treatment and cholesterol feeding may have neutralized each other; subsequent investigations will have to settle this point. In regard to parasitic infection, a slight recurrence of mange (G17., Dec. 13 to 20) appears to corroborate my previous findings, but another factor will also have to be considered, *viz.*, the influence of pregnancy in combination with Roentgen treatment and cholesterol feeding. This will be discussed briefly in connection with the third period.

Summarizing briefly the foregoing:

1. The increase in the cholesterol value of the blood resulting

from cholesterol feeding and the decrease resulting from mange observed in the first period has been confirmed by the findings in the second period.

2. The effect of the Roentgen rays cannot yet be determined definitely owing to the influence of intercurrent factors; the results suggest the possibility of a decrease of cholesterol values in the blood due to Roentgen treatment.

3. The effect of combined Roentgen treatment and cholesterol feeding suggests that these factors may neutralize each other, but the influence of intercurrent factors makes definite conclusions premature, and emphasizes the need of further investigations.

Third Period.—During this period the effect of castration was studied as an additional factor. The measure was suggested by the influence on malignant conditions of the "cancer age," *viz.*, the age at which reproductive activity decreases. It will be remembered that the principal aim of the experiment was the reproduction of malignant proliferation. One goat of each one of the three groups referred to in the discussion of the second period was castrated. Thus, in Group A (cholesterol feeding alone) G19 was castrated, and both goats belonging to this group continued to be fed 0.30 gm. of cholesterol daily; in Group B G16 was castrated, and both animals belonging to this group (G16 and G26) continued to be treated with the same doses of Roentgen rays; in Group C the male (G20) was castrated, and the combination of Roentgen treatment and cholesterol feeding was continued as before.

In order to verify previous observations on the changes in the cholesterol content caused by anesthesia, samples of the blood were taken from 10 to 15 minutes after the goats had been given ½ grain of morphine, before the administration of the anesthetic, and again on completion of the operation when the animal was still in narcosis. In every instance there was a decrease in the cholesterol content below the value obtained before the anesthetic was given and similar to the decrease observed in dogs (Fig. 4). An additional test was made with the blood of G16, taken 5 minutes after the beginning of anesthesia (the animal being completely unconscious) and demonstrated the fact, observed also in experiments on dogs, that anesthesia is at first accompanied by an increase of the cholesterol content, but that lower values

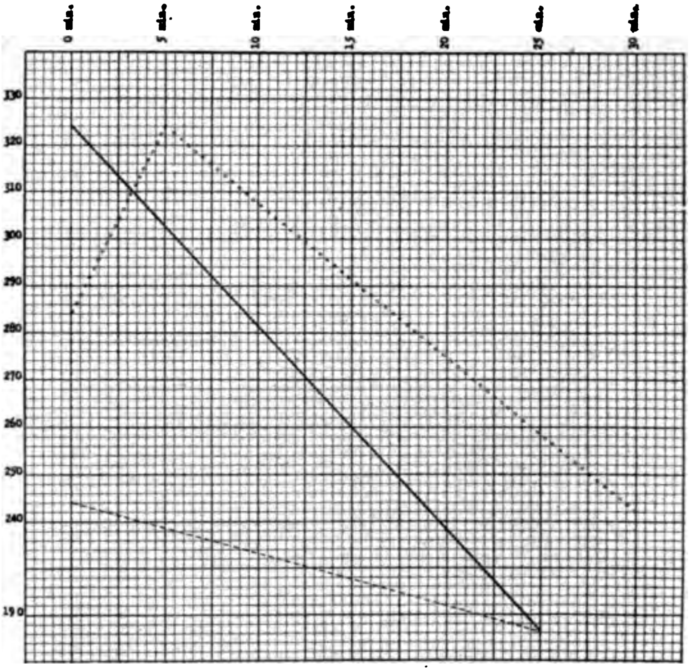


FIG. 4. The effect of anesthesia on cholesterol values. Goat 16, ° ° °, Goat 19, ———; Goat 20, — — —.

occur after the anesthetic has been given for some time. The length of this period appears to vary in different animals. Experiments on the effect of anesthesia on the cholesterol content of the blood are still in progress. The influence of the operative procedure itself need not be taken into consideration, since in our experiments on dogs the above results were obtained by means of the anesthetic alone.

The effect of castration is illustrated in Figs. 5a, 5b, and 5c. In Groups A and B increase of the cholesterol content following castration may be observed by comparing the values of the castrated and the non-castrated animal. In Group C the difference is less marked as the intercurrent factor of pregnancy in the animal intended for a control (G17) is likely to have increased the cholesterol values.

During the third period (Figs. 5a, 5b, and 5c) the highest cholesterol values of all were found in the blood of G19 (cholesterol-fed and castrated), the lowest values were found in the blood of G26 (no castration, Roentgen treatment only), while intermediate values could be observed in the blood of G16 (Roentgen treatment and castration).

It is further interesting to note that although the cholesterol value of G19 dropped as low as that of G20 (0.186 per cent) after anesthesia, the animal receiving cholesterol only went up to 0.330 per cent during the week following operation, whereas G20 which received cholesterol in combination with Roentgen treatment went down to 0.130 per cent.

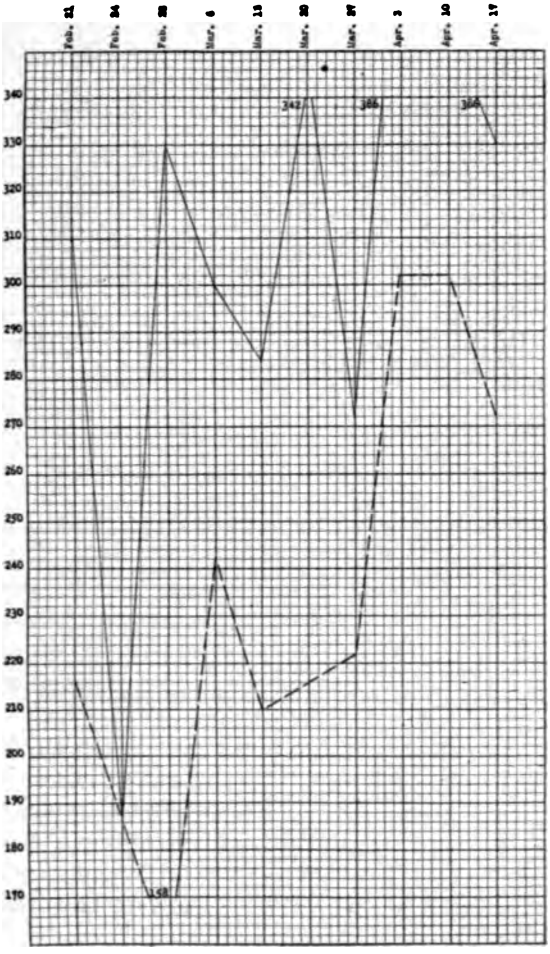


Fig. 5 a. Cholesterol values for Group A, Period III. The effect of cholesterol feeding (Goat 21, — — —) and of cholesterol feeding and castration (Goat 19, ———).

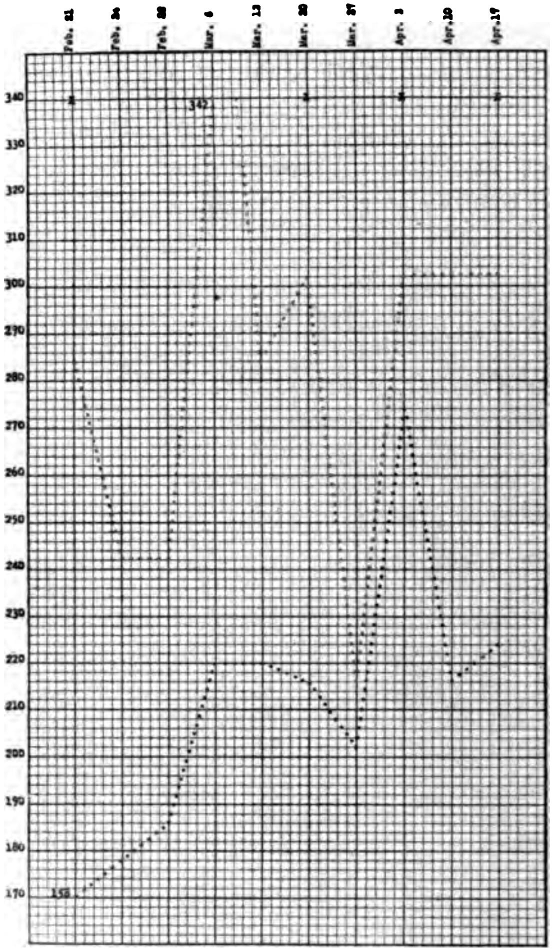
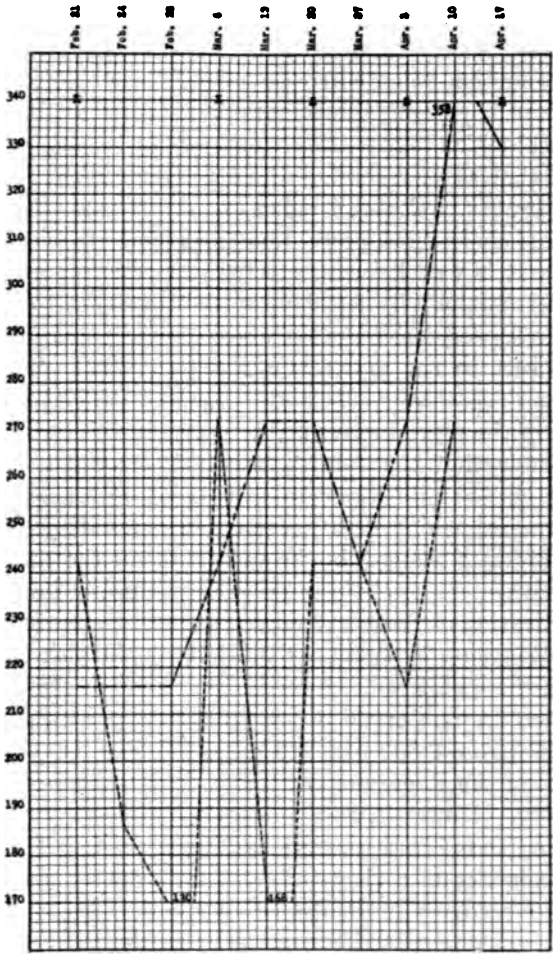


Fig. 5 b. Cholesterol values for Group B, Period III. The effect o Roentgen ray treatment (Goat 26, ...) and of Roentgen ray treatmen and castration (Goat 16, ° ° °). X indicates Roentgen ray treatment.



. 5 c. Cholesterol values for Group C, Period III. The effect of ·erol feeding, Roentgen ray treatment and pregnancy (Goat 17, —) and of cholesterol feeding, Roentgen ray treatment and castra- ɜoat 20, — — —). X indicates Roentgen ray treatment.

These observations seem to suggest that castration and cholesterol feeding both tend to increase the cholesterol content of the blood, but that the Roentgen rays, as has already been suggested, appear to have a depressing influence on the cholesterol values, the latter deduction being corroborated by the alternating effect of the week of Roentgen treatment (decrease) and the week of rest (increase) which became more marked during this period.

The fact that G26, receiving Roentgen treatment alone and having the lowest cholesterol value of all, appears to be slowly increasing his cholesterol percentage may perhaps be explained as follows. It is well known that the reproductive cells in the sex glands are particularly sensitive to the Roentgen rays and that animals can be castrated by Roentgen treatment alone. Prolonged exposure to the rays, even when extremely small doses are used, may therefore result in permanent injury, if not in the destruction of the reproductive cells.

It is possible that the total number of Roentgen ray units (approximately 150 milliampere minutes of diffuse treatment, wide open diaphragm) which G26 received in 5 months, may have sufficed to produce changes in the sex glands of the animal, and that the increase of the cholesterol values found in its blood are the first indication of these histologic changes. However, microscopic examination of the tissues will have to furnish conclusive evidence on this point.

The sudden drop observed in the cholesterol percentage of G16 on March 27 (from 0.302 to 0.216 per cent) may have been due to the effect of the Roentgen treatment during the week preceding this entry. However, as the animal had been lying down a good deal, appeared listless, and had been feeding badly for 3 days, the influence of a slight ailment followed by spontaneous and complete recovery must also be considered, especially as the cholesterol values remained constant during the following weeks.

A recent publication by Loewenthal (19) on the effect of castration on the blood of rabbits appears to corroborate my findings as regards the increase of the cholesterol content in the blood of goats.

The effect of pregnancy on the cholesterol values in women has been studied by Neumann and Herrmann (26), Aschoff (3), and Autenrieth and Funk (27); they report that an increase can be demonstrated at the beginning of the 5th month. Bacmeister and Havers (2) observed a similar change in the bile of pregnant bitches. As far as could be ascertained no other data have been published on the subject.

In my experiment the changes found in the blood of G17 during pregnancy are difficult to account for, because of additional factors (*i.e.*, cholesterol feeding, Roentgen treatment). On the whole, the values seem to be slightly higher. That the increase is not more pronounced may be due to the Roentgen treatment, to which the organism might be particularly sensitive during pregnancy. As in other instances my observations on this point will have to be verified by future experiments.

In the light of recent studies on the stimulation of cell division by cholesterol, the influence of pregnancy and of castration on the cholesterol content of the blood would seem to be significant; in pregnancy the increase of the cholesterol values might be interpreted as a physiologic measure calculated to stimulate cell division in the embryo, while in castration elimination of the reproductive organs (in which cell proliferation is more constant than in any other part of the body) might be expected to result in a surplus of unused cholesterol.

The results indicated by my observations during the third period may be summarized as follows:

1. Anesthesia appears to reduce the cholesterol content of the blood.
2. Castration tends to increase the cholesterol values; the increase of body fat observed in castrates and the fact that cholesterol is stored in the body fat are in accordance with the apparent effect of castration.
3. The highest cholesterol values were found in the castrated and cholesterol-fed goat, the lowest values in the animal that had not been castrated and had received diffuse Roentgen treatment only, while intermediate values occurred in the animal that had been castrated and treated with Roentgen rays. These facts seem to support the assumption that castration increases and Roentgen treatment tends to reduce the cholesterol content of the blood.
4. The slight but steady increase of cholesterol found in the blood of the goat which received diffuse Roentgen treatment

alone recalls the fact that the Roentgen rays may destroy th reproductive cells to an extent equalling castration, and suggest that the increase itself may be due to the destruction of the reproductive cells in this animal. The observation will have to b corroborated by microscopic findings.

5. The influence of pregnancy on the cholesterol content of th blood, though indicated, is not sufficiently marked to permit definite conclusions. Whereas in the other goats cholesterol feeding and Roentgen treatment appeared to neutralize each othe it is possible that pregnancy rendered this animal more sensitiv to the effect of the rays and that the latter factor preponderate and counteracted the influence of pregnancy. Future observations alone can furnish conclusive data.

Technical Details Concerning the Chemical Analysis of the Blood for Cholesterol.

The original Autenrieth-Funk (27) method was used at first to determine the cholesterol values of the blood in my experiments. Towards the middle of the second period parallel tests were made by Autenrieth's method and Bloor's (24) modification thereof, which was published at the time. Latterly Bloor's first modification has been used exclusively as it is much more simple and in many cases appears to give more accurate results. Bloor's (28) second modification has not been used; the omission of saponification in the latter method gives somewhat higher values, and it was thought advisable to retain one standard in this series of experiments.

The fault of Autenreith's method to which Bloor has called attention, the occurrence of a brownish tint in the reaction, making colorimetric comparison difficult, though also observed by me in samples of pathologic human blood, has not given me any trouble in the tests made with goat's blood. In the latter instances both methods gave identical values with only a few exceptions; when a difference occurred it never exceeded 0.025 per cent and the average values of a great number of readings by both methods have been recorded in the table (second period, Jan. 17 to Feb. 1).

The test for cholesterol is based on a specific color reaction;

on addition of acetic acid anhydride and sulfuric acid the colorless chloroform extract of the blood assumes a green tone, varying in intensity according to the amount of cholesterol present in the blood. The time required for the reaction, *viz.*, till the maximum color intensity is reached, in any test is 15 minutes, according to Autenrieth and Funk (27), and Bloor (24), the reagents being left in the dark at a constant temperature of 35–37°C. during that time. In the course of my investigations the following observations led me to modify the factors of time and temperature.

I found that the rapidity of the reaction varied considerably in different blood samples,[2] the maximum color value being reached much more quickly in one specimen of blood than in another under parallel conditions. As the exact chemical nature of the process by which the color reaction is caused is not known, the assumption seems warrantable that the presence of other chemical substances besides cholesterol may retard or accelerate the reaction. Every precaution has been taken to prevent recognizable factors (impurity of reagents, difference in temperature) from playing a part in the occurrence of these variations. To obtain data as accurate as possible by taking the occurrence of these variations into account, I have adopted the following procedure.

The test is left in the dark[3] at room temperature for 5 minutes only, after which it remains exposed to the light, and readings with the Autenrieth-Hellige colorimeter (control of the instrument with tests containing pure cholesterol) are made at intervals of 2 minutes until the maximum color intensity is reached. In some cases the maximum value was found after two readings, in others after ten to thirty readings; it remains constant for 10 minutes in some and for as long as 20 minutes in others, after which the color begins to fade, giving lower values. It is obvious that a test which reaches its maximum value after two readings or 4 minutes, and in which the maximum remains constant for 8 minutes only, will already have begun to fade when the first reading is made after 15 minutes, and that, therefore, the value obtained will be lower than would be warranted by the amount of cholesterol present in the blood. When the reaction is of the rapid type described above, it is advisable to make two, three, or even four tests with different portions of the same extract; the same maximum value will be constant for the same length of time in these different portions, and the figures recorded in the tables will represent maximum values which remained constant for at least four consecutive readings at 2 minute intervals each.

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[2] I tested over 400 different blood samples during the last 6 months, the cholesterol value being determined in normal human blood. in human blood under various pathological conditions, and in the blood of several species of experimental animals (dogs, goats, spermophiles). An average of ten readings was made for each test, resulting in a total of 4,000 readings.

[3] I have found an ordinary microscope case very serviceable as a "dark cupboard."

Another observation which caused me to adopt the above technique was that some tests assume a decidedly yellow-green tint (not the brown tone referred to by Bloor) when left in the dark longer than 5 minutes. This tint differs materially from the emerald green of the pure cholesterol reaction and makes accurate colorimetric reading difficult. I noticed, however, that the exact shade required for matching the control color (either pure cholesterol or the fluid of the Autenrieth-Hellige colorimeter) can be obtained by exposing yellowish tests to strong daylight (near a window); whereas tests that are slightly too blue-green can be corrected by putting them in the dark for a couple of minutes. The actual values are not affected by the change of tint.

DISCUSSION.

Although the data furnished by the observations reported in this paper are by no means conclusive, they seem to suggest problems worth studying, *viz.*, the effect of Roentgen rays on the cholesterol content of the blood in relation to malignant conditions, the influence of the organs of reproduction on cholesterol metabolism, and the effect of bacterial or parasitic infection. The fact that the primary object of my experiment was the study of possible factors in malignancy rather than the study of cholesterol values for themselves, will, I think, sufficiently explain the indeterminate nature of most of my deductions. It must be remembered also that the animals used for the experiment were young, healthy, and of a species endowed with a remarkable power of resistance and recuperation, having many means of safety at their disposal in the organs that regulate cholesterol metabolism. The adequacy of these organs, moreover, is bound to remain an unknown quantity in every animal as long as the animal is alive. Since, comparatively speaking, the study of cholesterol metabolism is a new field of investigation, data for comparison are scarce, if they are not entirely lacking, as is the case in experimental work of this kind done on goats. However, although my experiment is still in progress, some of the results obtained so far by measures calculated to disturb the cholesterol balance seem to be encouraging, and I hope to publish these shortly.

SUMMARY.

1. The average normal cholesterol content of the blood in goats, though not definitely established, appears to be from 0.186 to 0.216 per cent.
2. The cholesterol content of the blood can be increased by cholesterol feeding.
3. Diffuse Roentgen treatment appears to lower the cholesterol content of the blood, but conclusive evidence will have to be furnished by future experiments. The beneficial effect of Roentgen treatment in malignant conditions, the increase of the cholesterol content observed in the blood of patients suffering from malignant disease, and the stimulation of cell division by cholesterol demonstrated by recent experiments, suggest that the study of the influence of the Roentgen rays on the cholesterol content of the blood may be of considerable value.
4. Castration tends to increase the cholesterol values, which may explain the increase of body fat found in castrates and after the cessation of reproductive activity, since the storage of cholesterol in the body fat has been demonstrated.
5. The increase of the cholesterol content of the blood in pregnancy, reported by others, has been found indicated in my experiment, although intercurrent factors forbade definite conclusions.
6. The normal process of growth in the embryo caused by cell proliferation and the effect of cholesterol on cell division referred to above may explain the increase of the cholesterol content of the blood during pregnancy.

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THE RELATION OF OXIDASE REACTIONS TO CHANGES IN HYDROGEN ION CONCENTRATION.

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(Received for publication, September 11, 1916.)

Biological oxidations are known to be markedly affected by acids and alkalies. Loeb and Wasteneys[1] have shown that when sufficient HCl to give a hydrogen ion concentration of 1×10^{-4} M was added to unfertilized *Arbacia* eggs contained in a balanced salt solution only about half as much oxygen was absorbed as was absorbed by the same eggs when in neutral solution. On the other hand, when sufficient NaOH was added to give a hydroxyl ion concentration of 4.2×10^{-4} M the rate of oxygen absorption was approximately doubled; and when the hydroxyl ion concentration was increased to 8×10^{-4} two and three-fourths times more oxygen was absorbed than by the same eggs when in neutral solution. Similar results have been obtained in other cases.

It is a natural assumption that alkali directly influences the rate of oxidation. Several investigators have indicated that the oxidases are affected by changes in the acidity of the solution in which they act. Bertrand[2] found that laccase from *Rhus succedanea* acting on guaiacol was completely inhibited when sufficient H_2SO_4 was added to make the concentration 0.002 M. Abderhalden and Guggenheim[3] found that tyrosinase from various sources was destroyed by 0.01 M HCl and its action considerably retarded by 0.01 M NaOH. Wolff[4] has also pointed out that tyrosinase from *Russula delica* is most active when the solution is neutral to phenolphthalein.

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[1] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxvii, 410.

[2] Bertrand, G., and Muttermilch, *Compt. rend. Acad.*, 1907, cxliv, 1385.

[3] Abderhalden, E., and Guggenheim, M., *Z. physiol. Chem.*, 1907–08, liv, 331.

[4] Wolff, J., *Compt rend. Acad.*, 1909, cxlviii, 500.

In the experiments just mentioned the acidity or alkalinity was determined by the amount of standardized acid or alkali added to definite amounts of the solution containing the ferment, on the assumption that the dilution of the standardized acid by the mixture had the same effect as an equal dilution with distilled water.

The writer finds that this assumption is incorrect, as is clearly shown by determinations with a hydrogen electrode. The following example illustrates this.

An extract of potato (prepared by mixing equal volumes of grated potato and distilled water, allowing the mixture to stand for an hour, and filtering) was added to an equal volume of 0.01 M HCl. Since the acid was diluted to twice the original volume it would then be 0.005 M ($C_H = 5 \times 10^{-3}$) if the potato extract acted like distilled water. The hydrogen electrode measurement, however, showed the concentration to be only 5×10^{-4}. The presence of the potato has therefore reduced the acidity to one-tenth, although the extract itself is very nearly neutral. This effect is due to the protein present in the solution as well as the presence of other amphoteric electrolytes, including probably phosphates and carbonates.

In view of this observation it seemed desirable to repeat some of the earlier experiments to determine the real relation of the oxidases to acidity. A potato extract, prepared by grating the tuber with an equal volume of water and quickly filtering, was divided into several portions of 50 cc. each. These were placed in beakers, and sufficient HCl or NaOH was added to give the hydrogen ion concentration (as measured by the gas chain) shown in the first column of Table I. These were then exposed at room temperature and the rate of oxidation of the naturally occurring chromogen was followed by observing the appearance of red and finally black colors. It will be seen from the table that a hydrogen ion concentration of 5.5×10^{-4} was sufficient to inhibit the reaction completely.

Immediately after adding the acid to these extracts portions of 10 cc. were removed and 1 cc. of 2 per cent gum guaiac tincture was added to each. The results stated in the last column of Table I indicate that the same concentrations of acid which inhibited its reaction with the chromogen inhibit also the oxidation of gum guaiac.

TABLE I.

H ion concentration.	Oxidation of chromogen.		Oxidation of gum guaiac, 5 min.
	12 hrs.	24 hrs.	
1.5×10^{-3}	No color.	No color.	No color.
7.6×10^{-4}	“ “	“ “	“ “
5.5×10^{-4}	“ “	“ “	“ “
1.5×10^{-4}	Faint.	Red.	Blue.
7.5×10^{-5}	Red.	Black.	Deep blue.
2×10^{-7}	Deep red.	“	“ “
1×10^{-10}	Red.	“	“ “

To the three preparations, the oxidase action of which had been inhibited by contact with the acid for 12 hours, sufficient NaOH was added to neutralize to phenolphthalein. Portions of 10 cc. were then removed and mixed with gum guaiac, and the balance was exposed for another 12 hours. From the results stated in Table II it appears that the highest concentrations of acid destroyed the ferments, since neutralizing failed to revive them; the concentrations just sufficient to inhibit the oxidations, however, did not affect them irreversibly.

TABLE II.

Exposed for 12 hrs. H ion concentration.	Oxidation after neutralization.	
	Chromogen.	Gum guaiac.
1.5×10^{-3}	No color.	No color.
7.6×10^{-4}	Red.	Blue.
5.5×10^{-4}	“	“

Table III shows the results of an experiment with an extract of ripe Astrachan apples (prepared by grating the fruit with an equal volume of water and filtering). The extract was divided into several portions of 50 cc. each and sufficient acid or alkali was added to give the concentrations stated in the table (measured by the gas chain).

These results show that the concentrations of acid which are needed to inhibit oxidases are much lower than those stated by previous investigators; this is doubtless due to their faulty methods of measurement. Bertrand stated 0.002 M to be the lowest

TABLE III.

H ion concentration.	Oxidation.		
	Chromogen.		Gum guaiac.
	12 hrs.	48 hrs.	
1.6×10^{-3}	No color.	No color.	No color.
7.4×10^{-4}	" "	" "	" "
3.8×10^{-4}	Faint.	Faint.	Blue.
8×10^{-5}	Red.	Red.	Deep blue.
1.5×10^{-5}	"	Red-brown.	" "
1×10^{-7}	"	"	" "
2×10^{-9}	"	"	" "

concentration which completely inhibited oxidases and Colin and Sénéchal[5] report that horse-radish peroxidase had very little action in 0.004 M acid; while the present experiment indicates that 0.0005 to 0.007 M was sufficient to prevent the action of certain oxidases. It is therefore evident that the optimal activity of these oxidases is reached when they are in a medium which is very nearly neutral or slightly alkaline.

On this basis it may be possible to explain the remarkable activation of oxidation processes by the addition of exceedingly small amounts of alkali, as in the experiments of Loeb and Wasteneys on Echinoderm eggs. It is conceivable that in the unfertilized eggs the hydrogen ion concentration is too high for the rapid operation of oxidases, but that the added alkali changes this to the optimum condition.

All the ferments which have been investigated in this respect exhibit their maximum activity at a definite hydrogen ion concentration, and it was to be expected that the oxidases would behave similarly. Hudson[6] regards the activity of invertase (which behaves as an amphoteric electrolyte and hence is capable of combining with acids and bases) as proportional to the amount of ferment not so combined. This may apply to other ferments but it has not yet been shown to do so.

[5] Colin, H., and Sénéchal, A., *Compt. rend. Acad.*, 1911, cliii, 76; *Rev. gén. Bot.*, 1912, xxiv, 49.

[6] Hudson, C. S., *J. Am. Chem. Soc.*, 1910, xxxii, 1220.

PHOSPHATIDES IN THE DUCTLESS GLANDS.

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(Received for publication, September 5, 1916.)

This investigation was carried out to determine the amounts of phosphatides present in the ductless glands and the relation of the results to the functioning of these glands. Inasmuch as the work was of a preliminary nature no attempts were made to separate or isolate the groups of phosphatides from other extractive matters such as fat. Petroleum ether was chosen as a general solvent. Ordinary muscle fiber in the form of straight lean meat (chucks) was used as a standard, and brain tissue and spinal cord were employed for comparative purposes as examples of tissues exceptionally rich in phosphatides. Cattle glands are especially adapted for this work on account of their size and comparative freedom from adipose tissue. The following glands were used: Pineal gland from cattle; pituitary body, anterior and posterior lobes from cattle, and for comparative purposes also the anterior lobe from young calves; thyroid from cattle; thymus from young growing calves; suprarenals from cattle; and the true corpus luteum from pregnant cows. The thymus was selected from young calves 2 to 4 months old, the early growth period during which this gland is most active.

The various glands, from several hundred animals, were collected shortly after slaughtering and while still retaining the animal heat, and were carefully trimmed. In the case of the pituitary body, the outside capsule was discarded and the anterior and posterior lobes represent glandular tissue only. The corpora lutea were obtained by splitting the ovaries and removing the soft orange-colored glandular portion of the corpus luteum. The suprarenals also were skinned so that the portion employed represents the medulla and cortex only. The pineal and the thyroid were both trimmed as free as possible from all adherent connective and fatty tissues. The entire brain was employed, the outside tissue removed, and the brain washed with water until free from blood. The spinal cord represents the entire spinal marrow.

The various lots of glands and tissues were finely minced and desiccated *in vacuo* at 35–37°C. to constant weight, then coarsely ground and extracted with petroleum ether in a Soxhlet apparatus. After extraction, the various lots of petroleum ether containing the soluble portions of the glands were filtered and the solvent evaporated, first by a current of air and slight heating on a water bath and then by further drying *in vacuo* to constant weight. On these residues the total P_2O_5 was determined and the equivalent amounts of lecithin were obtained by multiplying the P_2O_5 figures by 11.24. These lecithin figures do not necessarily represent the actual amounts of lipoids present; they merely serve as a general guide for the present investigation. The phosphorus was determined on 1 gm. samples by boiling with 2 cc. of concentrated sulfuric acid and several small portions of nitric acid until oxidation was complete. The digested liquids were then diluted with water and the phosphorus was determined according to the official volumetric method.

In the tabulation will be found the loss of moisture and the percentage of petroleum ether-soluble substances in the fresh glands together with the yield of desiccated fat-free material, also the phosphorus in the extracted residue and the equivalent percentages of lecithin, both calculated on the basis of the residue and the fresh tissue.

The most striking feature of the tabulated figures is the fact that thyroid tissue contains no more phosphatides than ordinary muscle fiber, whereas all the other ductless glands show much larger amounts. The desiccated thyroid contained 0.41 per cent of iodine, indicating that the gland was exceedingly active physiologically and contained a maximum of active principles. This seems to be convincing evidence that the lipoids play no important part in the internal chemistry of the thyroid.

The petroleum ether-soluble portion of the muscle fiber as well as the thyroid consisted principally of fat. The color was light yellowish brown and the consistency that of ordinary beef fat.

					Petroleum ether-extracted material.				
	Moisture.	Petroleum ether-soluble.	Desiccated fat-free material.	Phosphatides calculated as lecithin in fresh tissue.	P_2O_5	Lecithin ($P_2O_5 \times 11.24$).	Consistency.	Color.	Odor.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*			
Pituitary body, anterior lobe, calves.....	81.00	2.30	16.70	1.44	5.57	62.61	Brain lecithin.	Yellowish brown.	Brain lecithin.
Pituitary body, anterior lobe, cattle.....	76.20	2.90	20.90	1.65	5.10	57.32	" "	" "	" "
Pituitary body, posterior lobe, cattle*...	82.60	1.80	15.60	0.96	4.74	53.28	" "	" "	" "
Pineal body, cattle.....	81.60	2.80	15.60	1.79	5.70	64.10	" "	" "	" "
Thyroid, cattle†.......	75.50	4.20	20.30	0.13	0.27	3.03	Semisolid, principally fat.	Light yellowish brown.	Beef fat, slight odor of lecithin.
Thymus, calves........	81.30	1.67	17.03	0.43	2.31	25.96	Semisolid, greasy.	Brownish yellow.	Lecithin.
Suprarenals, cattle‡...	75.40	4.43	20.17	2.37	4.75	53.39	Pasty.	Brownish red.	Crude lecithin.
Corpus luteum, pregnant cows..........	78.00	4.84	17.16	2.12	3.90	43.84	Semiliquid, pasty.	Reddish brown.	" "
Muscle fiber, lean meat.	74.20	1.44	24.36	0.14	0.85	9.55	Semisolid, principally fat.	Yellowish brown.	Beef fat, slight odor of lecithin.
Brain, cattle..........	76.90	10.78	12.32	5.70	4.70	52.83	Crude brain lecithin.	Light yellowish brown.	Brain lecithin.
Spinal cord, cattle.....	65.35	27.60	7.05	14.17	4.57	51.36	Crude brain lecithin.	Light yellowish brown.	" "

* Normal uterine-contracting power.

† 0.41 per cent iodine in desiccated glands.

‡ 0.9 per cent epinephrin in desiccated glands.

The infant thymus contained about three times as much phosphatides as lean meat from adult animals of the same species. The thymus fat was brownish yellow and possessed an odor resembling lecithin.

The anterior lobe of the pituitary body contained approximately ten times and the posterior lobe seven times as much phosphatides as straight lean meat. The posterior lobe showed the usual amount of uterine-contracting active principle, indicating normal physiological activity of the gland. The isolated uterus method as outlined by Roth[1] was employed in this work. The petroleum ether-soluble portion of this gland, both anterior and posterior lobes, resembled brain lecithin in consistency. The color was light yellowish brown and the odor similar to lecithin.

The pineal body showed about thirteen times more phosphatides than did muscle tissue. The petroleum ether-soluble portion resembled crude lecithin from brain tissue both in consistency, color, odor, and taste.

The corpus luteum of pregnancy was also rich in phosphatides, containing fifteen times as much as muscle tissue. The petroleum ether-soluble portion was of semisolid pasty consistency and reddish brown color, and possessed a strong lecithin odor.

The suprarenals were the richest in phosphatides of all the ductless glands, containing seventeen times more than ordinary muscle tissue. The petroleum ether-soluble portion was of pasty or waxy consistency, brownish red color, and pronounced lecithin odor.

The epinephrin content was determined by the manganese dioxide method described by Seidell.[2] The desiccated gland contained 0.9 per cent epinephrin, which means full physiological activity.

There was very little difference in the phosphorus contents of the extracted portions from brain and spinal cord, but the spinal cord contained almost three times as much petroleum ether-soluble substance as the brain. The soluble portions of the brain and spinal cord were both of waxy consistency, resembling soft paraffin, of light yellowish brown color, and characteristic lecithin odor.

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[1] Roth, G. B., *Bull. Hyg. Lab.*, No. 100, 1914.

[2] Seidell, A., *J. Biol. Chem.*, 1913, xv, 197.

There are no conclusive evidences that the pineal body and the thymus contain active principles of physiological significance. The corpus luteum has given some proof of physiological importance, but no active principle or means of standardizing the product for medicinal purposes have yet been discovered or outlined. In the pituitary body, the thyroid, and the suprarenals, however, we have definite means of judging the therapeutic activity of their internal secretions and it is from these glands that the present conclusions must be drawn. The analytical data show that these glands contain high amounts of active principles and consequently possess full physiological activity. The pituitary body and the suprarenals as well as the pineal, the thymus, and the corpus luteum of pregnancy contain considerable amounts of phosphatides in excess of ordinary muscle tissue and it is safe to assume, therefore, that the phosphatides play some part in the internal chemistry of all the ductless glands, with the exception of the thyroid.

SUMMARY.

The pituitary body and the suprarenals, as well as the pineal, the infant thymus gland, and the corpus luteum of pregnancy contain considerable amounts of phosphatides in excess of ordinary muscle tissue, indicating that the phosphatides play some part in the internal chemistry of these ductless glands. The thyroid does not contain any more phosphatides than straight lean meat and it may, therefore, be assumed that this gland performs its secretory functions independent of the phosphatides.

NUTRITION AND EVOLUTION.

SECOND NOTE.

By JACQUES LOEB and J. H. NORTHROP.

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(Received for publication, September 9, 1916.)

In a former paper Loeb[1] raised the question whether or not the evolution of animals as high in the scale of life as insects was possible without the existence of green plants. It is generally assumed in speculations on evolution that chlorophyll preceded the evolution of animals. Attacked from this point the question of evolution becomes a question of nutrition, or rather of the synthesis of the highly complex proteins of the body.

Loeb had found that sterile culture media like those used by Pasteur for the raising of yeast were also sufficient for the raising of flies—*Drosophila* was used for this purpose. It was pointed out that this did not prove that the power of synthesis of the flies is the same as that of the yeast since although the culture media were sterile, the flies were not; and that it was possible that the flies carried microorganisms which synthesized the proteins for them.[1] The question could only be decided by experiments with flies absolutely free from parasitic or symbiotic microorganisms.

Bogdanow[2] was the first to attempt to raise flies free from bacteria. He experimented on the blowfly, *Calliphora vomitoria.* His method consisted in sterilizing the eggs with solutions of $HgCl_2$. He found that sterile larvæ of the fly grew only very slowly on sterile meat, never reaching their full size. They died without undergoing metamorphosis. He assumes that they require the aid of bacteria for their normal nutrition. Wollman[3] repeated the experiments of Bogdanow, confirming his results. Delcourt and Guyénot[4] experimented with *Drosophila*. These authors first tried Bogdanow's method but did not succeed since the antiseptic treatment killed all the eggs of *Drosophila*. They proceeded by frequent transference of flies raised on acid media and thus finally obtained some colonies which on microscopic and bacteriological test proved sterile. Guyénot observed that sterile flies grew on yeast, living or dead, and that after several generations they also grew well on sterile potatoes. The larvæ grew well on peptone and salts but did not metamorphose.

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[1] Loeb, J., *J. Biol. Chem.*, 1915, xxiii, 431.

[2] Bogdanow, *Arch. Physiol., Suppl.*, 1908, 173.

[3] Wollman, E., *Ann. l'Inst. Pasteur*, 1911, xxv, 79.

We returned to the method of Bogdanow which seemed more direct and reliable, by raising sterile cultures from eggs sterilized in $HgCl_2$. Sterilized banana was covered with wet filter paper and female flies were put in the flasks with the banana. After several hours the eggs were taken from the filter paper and placed in 0.1 per cent $HgCl_2$ or a saturated solution of $HgCl_2$ in alcohol for 6 to 7 minutes. Most eggs are killed by the treatment but a small percentage recover and develop when put into a flask containing sterilized yeast and cotton. They grew and metamorphosed entirely normally on the following culture media:

Sterilized bakers' yeast	450 gm.
Water	1,000 cc.
Citric acid	50 gm.

The acid was added solely for the purpose of diminishing the chance of the development of bacteria when the flies were removed from the flask. The presence of citric acid diminished the number of larvæ (and probably also the number of eggs laid), since in the cultures without citric acid the number of larvæ and flies produced was greater. But cultures without citric acid usually became infected with bacteria when the old flies were removed from the flask for the purpose of starting new cultures. We have thus far raised twelve successive generations free from all microorganisms.

Our tests for the sterility of the flies consisted in putting the dead flies as well as parts of the culture into glucose agar, agar, plain broth, litmus milk, glucose broth, and potato tubes. All these tests remained permanently sterile. In addition, smears were made from the flies, pupæ, and larvæ, and examined. They were free from bacteria and fungi.

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[4] Delcourt, A., and Guyénot, E., *Bull. Sc. France et Belgique*, 1911, xlv, 249. Guyénot, E., *Compt. rend. Soc. biol.*, 1913, lxv, pt. i, 97, 178, 223, 270; 1914, lxvi, pt. i, 483, 548.

We made a large number of culture experiments with filter paper, cane sugar, $MgSO_4$, KH_2PO_4, NaCl, $CaCl_2$, and nitrogenous compounds as indicated in the following table. The sterile flies were transferred to the tubes containing the culture media with sterile pipettes, following the procedure of Guyénot in the manipulation of the flies.

Substrate.	Result.
Casein	Some larvæ reach full size but do not metamorphose.
Edestin	"
Egg albumin	"
Mixture of leucine, alanine, glycine, asparagine, tyrosine, tryptophane, and histidine[5]	"
Milk	"

Hence in none of these media was it possible to raise a single fly or pupa.

We then made experiments with sterile bananas and sterile potatoes. Bananas are the usual culture medium used for raising non-sterile *Drosophila* in large numbers. The results of our experiments on sterile bananas with sterilized flies were most remarkable. The larvæ grew slowly and very few pupated. Some larvæ remained alive 20 to 30 days. The flies which hatched from the pupæ were very small, about one-fourth the normal size, and had very little pigment. It was impossible to raise a second generation from them.

They were crossed with normal flies and cultivated on sterile yeast, but although both crosses were tried, no larvæ were obtained. When the females were normal flies and the males flies raised on sterile bananas, a few eggs were laid, but no larvæ hatched. This indicates that flies, free from microorganisms (yeast), when raised on sterile bananas or sterile potatoes were also sexually sterile. It is of course possible that this negative ult is not final although our conclusion is based on the result ten different cultures.

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[5] Some of these pure amino-acids were kindly given us by Dr. P. A. Levene.

Yeast was then the only medium on which normal larvæ could e raised with these sterile cultures of flies and it seems to be he indispensable food for these insects. Yeast must thereore contain some substance required for their growth and this substance must be rather resistant to heat since yeast heated for 1 hour at 120° is an excellent culture medium. We tried to isolate this substance from the yeast. Yeast extracted with boiling alcohol for 48 hours or with cold alcohol for 10 days was no longer able to serve as food. A mixture of the extracted yeast and of the extractive was no longer adequate to raise the larvæ, indicating an alteration of the necessary substance by the alcohol. The presence of traces of alcohol in this mixture in itself was not harmful since these flies grow normally in the presence of 2 to 3 per cent alcohol.

The necessary substance in yeast cannot be salts, or cane or grape sugar since non-sterile flies grow normally on Pasteur media, as Loeb's previous experiments have shown.

The addition of butter, nucleic acid, thymus or thyroid extract to the synthetic culture media mentioned in the table was also without effect. This fact, as well as the fact that the flies will not develop on sterile milk, shows that the necessary substance must be different from that needed for pigeons, rats, and other warm-blooded animals.

These observations show that an apparently polyphagous insect like *Drosophila* is in reality a monophagous insect, as yeast is a sufficient and perhaps the only sufficient food for them. They can live on any culture medium which can serve as a food for yeast with which they probably infect it. The experiments show that in the discussion of the synthetic power of the higher animals, the possible action of the microorganisms in the intestine must also be considered. As far as the origin of insects like *Drosophila* is concerned, their existence depends primarily on yeast.

THE INFLUENCE OF PARTURITION ON THE COMPOSITION AND PROPERTIES OF THE MILK AND MILK FAT OF THE COW.*

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(Received for publication, September 6, 1916.)

INTRODUCTION.

The question of variations in the composition of milk is a twofold one; one phase deals with variations in milk as secreted by the cow; the other phase deals with the changes in composition after the milk is secreted and before it is used for food. The latter phase of the question has in recent years overshadowed in large measure the question of the relation between the normal variation in composition to which milk is subject and its use as human food. The chemical side of this question has been for several years the object of critical study by the Missouri Agricultural Experiment Station. The results in regard to a number of the factors involved have already been published.[1]

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* Published by permission of the Director of the Missouri Agricultural Experiment Station. The data reported in this paper were collected in cooperation with the Dairy Division, United States Department of Agriculture, Bureau of Animal Industry. While the greater part of the data were taken the Dairy Division was represented by Leroy S. Palmer, who has been responsible for the preparation of the present paper.

[1] Eckles, C. H., and Shaw, R. H., Influence of the stage of lactation on the composition and properties of milk, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 155*, 1913; Influence of breed and individuality on composition and properties of milk, *ibid., Bull. 156*, 1913; Variations in composition and properties of milk from the individual cow, *ibid., Bull. 157*, 1913. Eckles, C. H., Influence of fatness of cow at parturition on per cent of fat in milk, *Missouri Agric. Exp. Station, Bull. 100*, 1912. Palmer, L. S., and Eckles, C. H., Carotin—the principal natural yellow pigment of milk fat, *J. Biol. Chem.*, 1914, xvii, 191, 211, 223, 237, 245; also *Missouri Agric. Exp. Station, Research Bull. 9, 10, 11*, and *12*, 1914. Eckles, C. H., and Palmer, L. S., Influence of plane of nutrition of cow upon composition

Investigations of other factors of importance influencing the normal composition of cows' milk are still in progress.

The general opinion prevails that cows' milk is not suitable for human food for a period of time after parturition. Opinion varies as to the length of time the milk is unfit for use, as low as 2 days and as high as 15 days being stated as the proper period by different authorities. The methods and standards for certified milk adopted by the American Association of Medical Commissions[2] place the figure at 7 days after parturition. The basis for the exclusion of cows' milk immediately after the birth of the calf is that its composition or constituents give rise to intestinal disorders. The evidence upon which this conclusion is based is, however, extremely difficult to find. The literature on colostrum milk is extensive,[3] but it is astonishingly free from specific data regarding the healthfulness or unhealthfulness of cows' milk immediately after parturition.

The data which are offered in the present paper indicate that colostrum milk is subject to variations in composition, or in other words that the effect of parturition on the composition of cows' milk may be greatly influenced by other factors. Data are presented on two questions: (1) the effect of milking the cow up to the time of parturition; (2) the influence of the length of the period the cow is dry before parturition.

Effect of Milking until Parturition, on Composition of Colostrum Milk and Milk Fat.

Milking the cow up to the time of parturition is not a common practice among dairymen. It is believed by these men that the dairy cow will produce more milk during the lactation period if given a rest of several weeks before parturition. Continuous

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and properties of milk and butter fat. Influence of overfeeding, *ibid.*, *Research Bull. 24*, 1914; Influence of underfeeding, *ibid.*, *Research Bull. 25*, 1916.

[2] *Proc. Sixth Ann. Conference, Am. Assn. Med. Milk Commissions*, 1912, 124.

[3] The literature on colostrum is reviewed very thoroughly by Weber, E., Untersuchungen über die Kolostralmilch der Kuh, der Ziege und des Schafes, *Milchwirt. Zentr.*, 1910, vi, pt. x, 433, pt. xi, 481, pt. xii, 543; also by Engel, St., Die Biochemie des Kolostrums, *Ergebn. Physiol.*, 1911, xi, 41–103.

milking of the cow is, however, a common practice in many localities. The effect of this practice on the composition of the colostrum is thus a matter of considerable interest, practical as well as scientific.

HISTORICAL.

We have been able to find only two references in regard to the influence of continuous milking on the colostrum milk. Nasse[4] many years ago stated that the change from colostrum to normal milk occurs very rapidly in the case of cows milked up to the time of parturition. Cook and Hills[5] reported the composition of the milk of a Jersey cow the night before and also a few hours after calving. The results as given by these authors are shown in Table I.

TABLE I.

*Composition of Milk before and after Calving.**

	Fat.	Protein.	Sugar.
	per cent	*per cent*	*per cent*
Before calving	10.18	6.35	4.92
After calving	7.48	6.48	5.18

* Cook and Hills.

The authors state that, "The cow instead of giving colostrum gave milk. . . . Under the microscope but few colostrum particles were found in the first milk." It is doubtful if the conclusion is justified that the milk after calving was normal, inasmuch as a protein percentage of 6.48 is abnormally high. The first milking was unquestionably less abnormal, however, than usually found for colostrum milk.

EXPERIMENTAL.

The data presented in the present paper were taken from three pure-bred Jersey cows in the University herd. Complete analyses of the milk and milk fat were made on composite samples taken at suitable intervals and covering a suitable length of time for a period both before and after parturition. The length of time covered by the composites varied, as indicated in each case in connection with the data.

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[4] Nasse, *Arch. Anat. Physiol. u. Wiss. Med.*, 1840, 259, cited by Weber, *Milchwirt. Zentr.*, 1910, vi, 440.

[5] Cook, W. W., and Hills, J. L., Abnormal Milks, *Vermont Agric. Exp. Station, 5th Ann. Report*, 1891, iii.

The analyses made included the specific gravity of the milk, the percentage of total solids, ash, fat, lactose, total protein, casein, albumin (heat-coagulable proteins), and in the case of two of the three animals the percentage of residual protein and residual non-protein nitrogen. The analyses of the milk fat included the saponification value, Reichert-Meissl number, iodine absorption value, and melting point.

The methods of analysis were largely those of the Association of Official Agricultural Chemists. In the fat analyses the Reichert-Meissl number was determined by the Leffman-Beam method, the iodine value by the Hübl method, and the melting point by Wiley's method. In the milk analyses the specific gravity was taken with the modified[6] Quevenne lactometer, the total solids were determined by the Babcock asbestos method, the fat in some cases was determined by the Babcock asbestos method but in others by the Babcock centrifugal method, the lactose by the optical method, using acid mercuric nitrate, the total protein and casein by the official A. O. A. C. methods, and the albumin (heat-coagulable proteins) by the provisional method of this Association. The residual protein was determined by the addition of 15 cc. of Almen's tannic acid reagent to the filtrate from the albumin determination. The precipitate was filtered off and treated like the albumin. The nitrogen remaining in the filtrate after the tannic acid precipitation was determined by evaporating the solution and treating in the usual way (Kjeldahl method). The nitrogen so determined was called the residual non-protein nitrogen. In some cases it was determined by difference between the total nitrogen and the total protein nitrogen.

The methods of taking the composites and the preparation of the milk and butter fat for analysis were practically identical with those described in detail elsewhere.[7] In the analyses made in 1913 the amount of formaldehyde used for preservation of the milk was increased from the earlier practice of one part in 5,000 to 2,500 to one part in 1,650.

The cows used in the investigation were in normal health and condition. The length of time since the last parturition was normal for each animal, being 408 days in the case of Cow 317, 375 days in the case of Cow 57, and 319 days in the case of Cow

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[6] Shaw, R. H., and Eckles, C. H., The estimation of total solids in milk by the use of formulas, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 134*, 1911, 16.

[7] Eckles and Shaw, *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 155*, 1913.

4. The character of the rations of the cows was not identical, but was uniform for the individual animals throughout the experimental period. Cows 317 and 57 were on pasture throughout, but received grain in addition, the latter consisting of a mixture of corn 4 parts, bran 2 parts, and linseed meal 1 part. Cow 4 was fed a grain mixture of the same character, but the roughage which she received consisted of corn silage and alfalfa hay.

RESULTS.

The analyses of the samples in the case of the three cows are shown in Tables III, IV, and V.

Before considering the effects of continuous milking on the composition of the colostrum milk in the case of the three cows it is necessary to have in mind the composition of colostrum when the cow is dry for a period of time before parturition; in other words, normal colostrum milk. Unfortunately, for purposes of the present discussion normal colostrum milk is not uniform in composition with different animals. As has been expressed by other authors, the difficulty arises in that the numerous analyses of colostrum milk which have been made have not been uniform with respect to the length of time after parturition the sample was taken for analysis. The reason for the lack of uniformity of colostrum analyses becomes apparent when the rapidity with which milk changes in composition after parturition is taken into consideration.

In general, however, colostrum milk is characterized by abnormally high specific gravity and percentage of total solids, ash, and protein, and by an abnormally low percentage of lactose. The fat varies, as reported by different investigators, some reporting high fat percentages, others normal, and still others low. The distribution of the proteins of normal colostrum appears to be characteristic, all the protein being present in amounts above normal, the chief characteristic being that the heat-coagulable proteins greatly predominate over the casein, which is the reverse of normal cows' milk. It also appears to be well established that the heat-coagulable proteins of colostrum consist largely of globulin, which is present in normal cows' milk in smaller proportion than albumin. The fat of colostrum milk is characterized by a low Reichert-Meissl number and saponification value, and a high iodine value and melting point. Colostrum is also characterized by a high content of epithelial cells, their peculiar grouping in the milk giving rise to the name "colostrum corpuscles." Colostrum is also characterized by its high yellow color, which has been found by us[8] to be due merely to a high concentration of carotin, the normal pigment of the milk fat of the cow.

The general characteristics of colostrum as stated in the preceding paragraph are shown in tabular form in Table II, minimum, maximum, and average values being given as found by various investigators.

The data in Tables III, IV, and V show examples of colostrum milk widely different in composition from normal colostrum as given in Table II, the milk from Cows 317 and 57 showing a greater difference than that from Cow 4.

TABLE II.

Composition of Normal Colostrum Milk.

	Minimum.	Maximum.	Average.*
Specific gravity	1.032	1.090	1.068
	per cent	*per cent*	*per cent*
Total solids	16.18	37.21	28.30
Ash	0.85	2.31	1.78
Total protein	5.72	27.33	15.85
Casein	2.64	7.59	4.82
Albumin	0.25	2.00	
Globulin	2.35	17.51	
Heat-coagulable proteins	5.06	20.21	15.85
Lactose	1.02	3.50	2.48
Fat	0.15	12.00	3.37**
Saponification value	209.5	226.4	
Reichert-Meissl number	6.16	26.0	
Iodine value	36.8	46.4	

* The average values are those reported by Eugling, who made analyses from twenty-three cows (*Ber. Tätigkeit landw. Versuchs. Vorarlberg*, 1878, ii, 33).

** Average of 51 analyses made by König (*Chem. mensch. Nahr. u. Genussmittel*, 1904, ii, 603) showed 3.97 per cent fat.

Considering the results in detail we find that parturition failed to exert the usual depressing influence on the percentage of lactose in each of the three cases, although it was somewhat below

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[8] Palmer and Eckles, *J. Biol. Chem.*, 1914, xvii, 199.

TABLE III.

Effect of Milking until Parturition, on Composition and Properties of Colostrum Milk and Milk Fat. Cow 317.

July 21 to Aug. 26, 1913.

Date.	Milk per day.	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.	Casein.	Heat-coagulable proteins.	Residual proteins.	Residual* non-protein nitrogen.	Percentage of total protein present as			Fat.			
												Casein.	Heat-coagulable.	Residual.	Saponification value.	Reichert-Meissl No.	Iodine value (Hübl).	Melting point.
1913	*lbs.*	*1.0—*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*							*°C.*
July 21–23	14.1	337	13.34	0.71	4.61	4.23	3.78	2.62	0.81	0.35	0.024	69.3	21.4	9.3	228.5	29.81	34.57	33.20
" 24–26	14.1	335	13.51	0.72	4.68	4.35	3.76	2.70	0.68	0.38	0.058	71.8	18.1	10.2	227.9	27.54	34.87	30.70
" 27–29	13.5	332	13.29	0.72	4.55	4.15	3.87	2.77	0.71	0.39	0.057	71.6	18.4	10.0	226.1	28.59	36.04	31.90
" 30–Aug. 1	12.3	348	13.62	0.73	4.57	4.18	4.14	2.81	0.97	0.36	0.051	67.9	23.4	8.7	226.3	27.17	36.63	32.60
Aug. 2, a.m.**	7.4	373	13.47	0.77	4.51	3.58	4.61	2.86	1.35	0.40	0.055	62.0	29.3	8.7	224.7	27.11	37.00	34.50
Parturition at noon.																		
Aug. 2, p.m.	6.5	368	14.01	0.78	4.43	4.00	4.80	2.72	1.70	0.38	0.045	58.8	35.4	5.8	223.0	24.43	37.09	34.10
" 3	15.5	367	12.22	0.80	4.31	2.78	4.33	2.82	1.13	0.38	0.064	65.1	26.1	8.8	221.1	22.50	38.14	34.80
" 5	18.1	331	13.61	0.82	4.39	4.20	3.77	2.74	0.63	0.40	0.065	72.7	16.7	10.6	220.3	23.83	40.17	34.30
" 7	23.4	328	13.86	0.75	4.48	4.60	3.71	2.75	0.56	0.40	0.050	74.2	15.1	10.7	223.3	25.14	38.34	37.10
" 9	21.5	319	12.65	0.76	4.20	3.95	3.41	2.54	0.43	0.44	0.042	74.5	12.6	12.9	222.6	26.52	37.68	36.80
" 11–12	21.5	319	12.38	0.75	4.40	3.70	3.28	2.46	0.44	0.38	0.039	75.0	13.4	11.6	222.4	27.74	36.71	35.40
" 13–14	22.1	318	12.48	0.74	4.44	3.80	3.22	2.45	0.39	0.38	0.044	76.1	12.1	11.8	222.7	26.55	38.29	37.20
" 15–16	21.9	316	12.47	0.71	4.40	3.95	3.18	2.36	0.44	0.38	0.038	74.2	13.8	12.0	227.7	26.55	38.46	36.90
" 17–18	24.3	317	12.38	0.71	4.47	3.75	3.18	2.45	0.37	0.36	0.042	77.0	11.6	11.4	224.9	29.47	36.42	34.90
" 25–26	25.6	314	11.98	0.70	4.43	3.60	3.00	2.29	0.38	0.33	0.040	76.3	12.7	11.0	226.8	30.42	34.43	35.20

* Difference between total nitrogen and total protein nitrogen.

** Length of lactation period ending Aug. 2, 408 days.

TABLE IV.

Effect of Milking until Parturition, on Composition and Properties of Colostrum Milk and Milk Fat. Cow 57. July 12 to Sept. 3, 1913.

Date.	Milk per day.	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.	Casein.	Heat-coagulable proteins.	Residual proteins.	Residual* non-protein nitrogen.	Percentage of total protein present as			Fat.			
												Casein.	Heat-coagulable.	Residual.	Saponification value.	Reichert-Meissl No.	Iodine value (Hübl).	Melting point.
1913	lbs.	1.0—	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent							°C.
July 12–14	5.1	338	15.45	0.80	4.50	4.90	4.89	3.37	1.11	0.41	0.063	68.9	22.7	8.4	220.6	25.60	36.96	37.2
" 15–17	5.2	304	15.02	0.80	4.38	4.63	4.92	3.37	1.18	0.37	0.047	68.5	24.0	7.5	223.1	27.62	35.77	37.6
" 18–20	6.1	342	14.61	0.81	4.60	4.10	4.76	3.27	1.10	0.39	0.054	68.7	23.1	8.2	225.7	28.62	35.08	37.3
" 21–23	6.3	366	14.75	0.80	4.60	4.40	4.67	3.28	1.01	0.38	0.044	70.2	21.6	8.2	226.8	29.58	34.43	37.7
" 24–26	5.9	367	14.97	0.79	4.78	4.55	4.54	3.15	1.00	0.39	0:048	69.4	22.0	8.6	226.0	29.92	34.91	37.9
" 27–29	5.8	352	14.94	0.77	4.55	4.55	4.70	3.20	1.10	0.40	0.059	68.1	23.4	8.5	225.3	29.27	35.64	38.2
" 31–Aug. 1	5.4	362	14.79	0.80	4.34	4.50	4.85	3.21	1.21	0.43	0.048	66.1	25.0	8.9	225.5	29.42	35.87	38.3
Aug. 2– 4	5.2	368	14.61	0.76	4.38	4.22	4.87	3.14	1.31	0.42	0.060	64.5	26.9	8.6	226.2	27.91	36.50	37.0
" 5– 7	6.1	370	14.41	0.76	4.36	3.98	4.86	3.05	1.37	0.44	0.072	62.8	28.2	9.0	224.5	27.72	35.10	35.0
" 8–10	7.3	376	14.54	0.75	4.76	3.75	4.90	3.06	1.46	0.38	0.061	62.5	29.8	7.7	225.1	27.25	35.81	35.8
" 11, a.m.**	4.5	380	13.56	0.80	4.29	2.78	5.26	3.02	1.82	0.42	0.068	57.4	34.6	8.0				
Parturition at noon.																		
Aug. 11, p.m.	3.9	380	14.86	0.80	4.62	3.78	5.18	3.04	1.81	0.33	0.078	58.7	34.9	6.4				
" 12	17.2	378	13.28	0.77	5.06	2.65	4.32	3.06	0.91	0.35	0.075	70.8	21.0	8.1	227.9	27.23	35.05	33.3
" 13	26.4	356	13.25	0.79	4.72	3.35	4.21	3.02	0.81	0.38	0.039	71.8	19.2	9.0	227.0	27.28	34.45	32.8
" 15	23.6	350	13.19	0.77	5.10	4.17	3.88	3.10	0.36	0.42	0.042	79.9	9.3	10.8	226.7	31.17	37.09	35.8
" 18	27.7	342	13.63	0.76	5.10	3.85	3.65	2.90	0.32	0.43	0.048	79.5	8.8	11.8	229.7	34.89	35.87	35.0
" 20	25.3	329	13.94	0.72	5.10	4.58	3.27	2.60	0.31	0.36	0.041	79.5	9.5	11.0	224.6	30.76	37.76	36.0
" 22–23	26.9	334	14.37	0.73	5.16	5.00	3.32	2.72	0.19	0.41	0.023	81.9	5.7	12.4	224.3	31.61	40.04	34.9
" 24–25	27.4	333	14.08	0.73	5.19	4.60	3.34	2.71	0.22	0.41	0.033	81.1	6.6	12.3	226.7	32.01	39.36	33.8
" 26–27	27.2	333	14.08	0.71	5.15	4.60	3.37	2.67	0.17	0.53	0.022	79.3	5.0	15.7	225.7	31.45	38.22	34.1
Sept. 2– 3	26.6	322	13.86	0.68	5.16	4.58	3.23	2.25	0.34	0.64	0.030	69.7	10.5	19.8	228.5	30.59	35.40	31.0

perties of Colostrum Milk and Milk Fat. Cow
Sept. 17 to Oct. 12, 1907.

Date.	Milk per day.*	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.*	Casein.	Heat-coagulable proteins.	Percentage of total protein present as		Fat.			
										Casein.	Heat-coagulable.	Saponification value.	Reichert-Meissl No.	Iodine value (Hübl.)	Melting point.
1907	lbs.	1.0—	per cent	per cent	per cent	per cent	per cent	per cent	per cent						°C.
Sept. 17	12.1	346	15.83		4.50	5.36	4.85	3.70	0.70	76.3	14.4	231.4	23.97	32.43	32.70
" 20	8.6	362	17.77		4.68	6.38	5.42	4.40	0.83	81.1	15.3	234.1	25.69	32.87	32.85
" 23	11.3	360	16.94		4.79	6.04	5.17	3.95	0.89	77.9	17.2	231.3	25.28	33.51	33.20
" 26	10.7	376	17.58		5.04	5.96	5.68	4.27	0.83	76.6	14.6		13.88	30.39	34.35
" 29	9.9	390	18.11		4.64	5.82	6.19	4.66	1.02	75.3	16.5	217.4	10.75	36.16	34.05
Oct. 2	9.9	420	19.16		3.95	6.30	7.02						8.90	32.03	37.03
" 4**	11.3	445	18.48		4.30	5.90	6.57	4.85	0.96	73.8	14.4				
Parturition.															
Oct. 5 a.m.	13.8	330	20.88	0.87	4.59	7.20	7.02	4.47	0.89	63.7	12.7				
p.m.		330	20.32			8.50	6.51	4.34	0.70	66.7	10.8	221.3	19.14	30.02	38.13
" 6 a.m.	22.3	330	15.92			5.00	5.74	4.34	0.51	75.6	8.9	222.1	17.76	31.13	37.33
p.m.		310	16.36		4.80	6.06	5.10	3.64	0.43	71.4	8.4	224.1	18.57	33.18	36.35
" 7 a.m.	27.7	310	16.32	0.74	5.71	6.05	4.72	3.83	0.34	81.1	7.2	222.7	19.96	31.32	36.03
p.m.		340	16.87		5.35	6.25	4.53	3.38	0.61	74.6	13.5	224.9	22.13	31.30	36.18
" 8 a.m.	26.3	335	15.82	0.78	5.72	5.36	4.91	3.77	0.50	76.8	10.2	224.3	22.76	32.69	35.25
p.m.		350	15.25	0.81	5.22	5.32	4.59	3.57	0.54	77.8	11.8	225.0	23.19	33.21	35.50
" 9 a.m.	30.1	355	14.84	0.79	5.48	4.89	4.40	3.45	0.53	78.4	12.1	228.1	24.13	32.26	34.60
p.m.		325	15.33	0.82	5.35	5.31	4.20	3.32	0.51	79.1	12.1	226.2	23.36	33.51	34.65
" 10 a.m.	29.7	340	14.84	0.76	5.53	4.83	4.40	3.38	0.59	76.8	13.4	224.8	25.29	33.76	34.48
p.m.		335			5.65							226.5	24.37	34.62	34.45
" 11 a.m.	28.6	340	14.96	0.74	5.67	5.16	4.47	3.13	0.42	70.1	9.4	225.3	25.21		33.70
p.m.		350	13.92	0.81	5.35	4.31	4.21	3.06	0.43	72.8	10.2	220.6	24.23	34.84	33.08
" 12	31.2	335	14.28	0.78	5.42	4.60	4.15	3.00	0.43	72.4	10.4	228.2	24.68	33.92	33.68

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* Total N ×6.38.

normal in the case of Cows 57 and 4. In regard to the casein we find no effect in the case of Cows 317 and 57, but a high casein percentage for Cow 4. The composition of the milk fat was uniformly unaffected by parturition in the case of each of the three cows. Very abnormal fat constants followed parturition in the case of Cow 4, but it is open to question whether this was the result of parturition, inasmuch as the fat showed the same abnormalities for several days before parturition in even more striking degree.

The most uniform effect of parturition was upon the heat-coagulable proteins; namely, the albumin and globulin of the milk. The percentage of these proteins increased very materially up to the time of parturition, reaching a maximum in the first milk drawn after parturition. The proportion of the total protein of the milk attained at this time was remarkably great for Cows 317 and 57, being nearly 35 per cent in each case. In spite of this great increase in albumin and globulin the actual proportion of the total milk represented by these proteins was far below the minimum value for heat-coagulable proteins of normal colostrum as given in Table II.

As already pointed out, the results in the case of Cow 4 were less striking than for the other two cows. The authors attribute this to the fact that the milk and milk fat of this cow showed the characteristics of the end of the lactation period before parturition occurred.

The general conclusion which we believe is justified by the data given for the three cows under discussion is that when a cow is milked up to parturition the colostrum milk will follow closely the composition of the milk given before parturition, with the exception of a more or less marked increase in the heat-coagulable proteins. If the composition of the milk and milk fat shows the characteristics of the end of the lactation period before parturition, the colostrum milk and milk fat will also show these characteristics. If the milk and milk fat are normal in composition before parturition the colostrum milk and milk fat will also be normal, with the exception already noted.

nfluence of Length of Period of Dryness of Cow, on Composition of Colostrum Milk and Milk Fat.

The data given in the foregoing pages of this paper indicate learly that the abnormal composition of milk and milk fat usually haracteristic of colostrum is greatly lessened when the cow is ilked up to parturition, and may be evident only to a slight deree in the case of a heavy milking cow. This result raises the uestion whether the length of time the cow is dry before parurition influences the composition of colostrum milk. Wintertein and Strickler[9] have suggested that this factor has a great nfluence on the properties of colostrum, greater than the influence ither of breed or manner of feeding. St. Engel,[3] however, uestions whether this viewpoint can be upheld. Neither inestigator offered any data in support of his opinion. Certain ata which we have collected in connection with our colostrum tudies and which seem to have a bearing on this point are given n Table VI. This presents complete analyses of the milk and milk fat for the first two or three milkings of four cows, following parturition. The length of the period of dryness of the cows varied from 19 to 83 days. The animals were all pure-bred dairy cows, on pasture, and probably received some additional grain, which is the usual practice in the dairy herd of the Missouri Agricultural Experiment Station. Our records, however, are not complete on this point in connection with these cows. The methods of analysis, handling of cows, etc., were identical with the procedure described in the first part of the paper in connection with the other data reported.

In the case of each of the animals the first milk was drawn within a few hours after parturition. The animals having been dry for a period of time, the quantity obtained was not sufficient in all cases for complete analyses of both milk and milk fat. Such data as it was possible to secure are reported in the table. Complete analyses were made in each case as soon as sufficient milk was obtained.

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[9] Winterstein, E., and Strickler, E., Die chemische Zusammensetzung des Colostrums mit besonderer Berücksichtigung der Eiweisstoffe, *Z. physiol. Chem.*, 1906, xlvii, 58.

TABLE VI.

Influence of Period of Dryness of Cow, on Composition and Properties of Colostrum Milk and Milk Fat.

Cow.	Period dry.	Date.	Milk per day.	Sp. gr.	Total solids.	Ash.	Lactose.	Fat.	Total proteins.	Casein.	Heat-coagulable proteins.	Residual protein.	Residual non-protein nitrogen.	Percentage of total protein as Casein.	Percentage of total protein as Heat-coagulable.	Percentage of total protein as Residual.	Fat. Saponification value.	Fat. Reichert-Meissl No.	Fat. Iodine value (Hübl).	Fat. Melting point.
	days		*lbs.*	*1.0—*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*							*°C.*
		1913																		
22*......	19	July 5, a.m.	4.6	345		0.71		4.43	6.64	4.02	1.86	0.76	0.055	60.6	28.0	11.4				
		" 5, p.m.		342	13.08	0.75	4.13	3.95	5.01	3.45	1.07	0.49	0.045	68.8	21.4	9.8				
		" 6	7.0	350	13.09	0.81	4.28	3.10	4.45	3.29	0.75	0.41	0.041	74.0	16.8	9.2	222.2	21.14	33.14	35.9
		1908																		
205**....	20	Aug. 27, a.m.		510	17.44		2.62	2.85	9.95	4.66	4.66			46.8	46.8					
		" 27, p.m.	1.9	372	12.51	0.83	3.60	1.98	6.38	3.77	1.40			59.1	21.9			27.07		35.9
		1913																		
17†......	30	May 30, a.m.				0.84														
		" 30, p.m.		343	13.72	0.83	4.70	2.80	5.11	3.68	0.94	0.49	0.042	72.0	18.4	9.6				
		" 31	14.7	334	13.17	0.84	4.72	2.63	4.69	3.56	0.66	0.47	0.035	75.9	14.1	10.0	224.6	21.50	34.89	35.8
		1908																		
400‡.....	83	Oct. 23, a.m.		366	22.22	0.80	2.65	1.30	14.42	7.90	5.53			54.7	38.3				30.33	38.6
		" 23, p.m.		345	15.78	0.95	3.81	3.26	7.91	5.30	1.96			67.0	24.8		223.6	21.92	25.65	39.7

* Parturition during night of July 4.

** Parturition during early morning of Aug. 27.

† Parturition during night of May 29.

‡ Parturition during night of Oct. 22

DISCUSSION OF RESULTS.

Taking into account the fact that the first milking after parturition, that is, the true colostrum, in the case of Cow 17 was not sufficient for any analysis save that of percentage of ash, the data in Table VI show the interesting fact that the abnormalities in composition of colostrum milk are in direct relation to the length of the period of dryness. All the colostrum samples showed a composition more or less characteristic of the first milk drawn after parturition, but this was particularly striking in the case of Cow 400, which was dry 83 days. The percentage of total solids, protein, casein, heat-coagulable proteins, and ash was abnormally high in each case, while the percentage of lactose and fat was abnormally low. However, in the case of Cow 22, dry 19 days, the milk was much less abnormal in composition, although it showed a protein, casein, and heat-coagulable protein content considerably above normal. The fat percentage of the colostrum milk of Cow 17, namely 4.43, which was only slightly below the normal fat percentage of this cows' milk (which was 4.8 per cent) is in striking contrast to the extremely low fat percentage of the colostrum milk of Cow 400.

The data in Table VI are less complete in regard to the physical and chemical constants of the colostrum fat in the case of the four cows, owing to the fact, already mentioned, that there was insufficient milk to furnish enough fat for these analyses. The data available indicate, however, that the length of the period of dryness had little, if any, influence on the composition of the colostrum fat. In each case the fat showed the composition characteristic of colostrum fat, having a low saponification and Reichert-Meissl value and high melting point. The iodine value of the fats analyzed did not show the extremely high figure usually characteristic of colostrum fat.

The data in Table VI in general lead to the conclusion that the length of the period of dryness is a factor influencing the composition of colostrum milk, but does not influence the composition of colostrum milk fat. The shorter the time the cow is dry before parturition the less abnormal will be the colostrum milk.

SUMMARY.

The conclusions which we have drawn from the data presented in the present paper may be summarized as follows:

1. Parturition in the case of the cow is normally accompanied by the production of milk of extremely abnormal composition, called colostrum.

2. When cows are milked up to parturition, however, the colostrum milk and milk fat are much less abnormal in composition and follow closely the composition of the milk and milk fat given before parturition.

3. The chief characteristic of the milk as the cow approaches parturition in these cases is the marked increase in the content of heat-coagulable proteins, which reach their maximum in the first milk following parturition, that is, the true colostrum milk. This would indicate that a high content of heat-coagulable proteins is the chief, if not the only, real effect of parturition on the milk.

4. The length of time the cow is dry before parturition is a factor influencing the composition of colostrum milk, but does not influence the composition of colostrum milk fat. The shorter the time the cow is dry the less abnormal will be the composition of the colostrum milk.

A METHOD FOR THE DETERMINATION OF NITRIC NITROGEN.*

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A simple and accurate method of determining nitric nitrogen in culture media and soil extracts has been much needed by soil biologists and chemists. A procedure to be satisfactory must yield reliable results in the presence of unstable organic substances like the amino and amide compounds.

Practically all of the methods now in use involve the reduction of the nitrate to ammonia and its subsequent distillation into standard acid. The reduction is effected by nascent hydrogen which is generated either in an acid or an alkaline solution. The principal objection to the methods using an acid solution is that they take too long to effect reduction, or if the time is shortened by using larger quantities of reagents, the increased amount of acid may hydrolyze some organic material. The better planned methods using an alkaline solution accomplish the reduction during distillation so that much less time is needed to obtain results, but the amount of alkali required is so great that unstable organic matter may be decomposed.

Organic material does not affect the results obtained by the proposed procedure. The method is accurate and requires only the simplest modification of the regular Kjeldahl apparatus. A zinc-copper couple is prepared in such a way that it can be renewed very easily. This couple decomposes water at the boiling point. The hydrogen is evolved and the oxygen of the water combines with the zinc to form zinc oxide. The advantage in the use of this

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* Published by permission of the Secretary of Agriculture.

Thanks are due Mr. F. L. Goll of the Office of Soil Bacteriology for the illustration accompanying this report.

couple is that neither an acid nor an alkaline reaction is necessary to promote the generation of hydrogen. Therefore only a small amount of fixed alkali is required to expel the ammonia formed by the reduction.

HISTORICAL.

The fact that a zinc-copper couple has the power of decomposing pure distilled water at room temperature has not been generally known and this undoubtedly is the reason that a direct distillation method using this couple has not been perfected for the determination of nitrates in soil analyses.

M. W. Williams[1] showed that dilute solutions of nitrate are completely reduced to ammonia by this couple if kept at 30°C. for a few hours, and that the reduction is hastened by the presence of carbonic acid, and a neutral electrolyte like sodium chloride. He poured the solution containing the ammonia into a Nessler tube and titrated with Nessler's reagent in the ordinary way.

Keating Stock[2] used granulated zinc for his couple and reduced the nitrate solution at 20–25°C. for 48 hours. He distilled off the ammonia and Nesslerized. This method is slower than most of the acid methods, but it does not take any longer than the acetic acid method with this same couple that is used at the Rothamsted Station.[3]

Apparatus.

The apparatus designed for use with this method is the simplest one that will satisfactorily scrub the distillate before the ammonia formed by the reduction passes into the standard acid. The apparatus may be understood by referring to Fig. 1.

The receiving end of the Wagner form of connection tube is fitted with an extra long rubber stopper[4] (A) which is slipped almost up to the bulb.

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[1] Williams, M. W., *Tr. Chem. Soc.*, 1881, xxxix, 100; *Analyst*, 1881, vi, 36.

[2] Stock, K., cited in Sutton, F., Volumetric Analysis, Philadelphia, 10th edition, 1911, 468.

[3] Russell, E. J., *J. Agric. Sc.*, 1914, vi, 18.

[4] A. H. Thomas Co.'s No. 25 (28 mm. bottom by 34 mm. top and 38 mm. deep).

A large size lipless test-tube (B) about 23 mm. in diameter by 150 mm. in length is fitted with a rubber stopper (C) containing two holes, one for the receiving end of the connection tube, and the other for the glass tube (D) which goes to within 1 cm. of the bottom of the test-tube. The holes in the rubber stopper should be made rather small for the tubes to insure a tight joint. If the tubes are heated a little before they are finally put in position they will stick firmly to the stopper. If the bottom of the stopper when pushed as far as possible into the test-tube does not press firmly against the sides of the tube it must be cut off to the place where its sides do press against the tube. When steam gets between the test-tube and the stopper it will soon work to the top and the stopper will have to be replaced. The joint (E) between the test-tube and the rubber

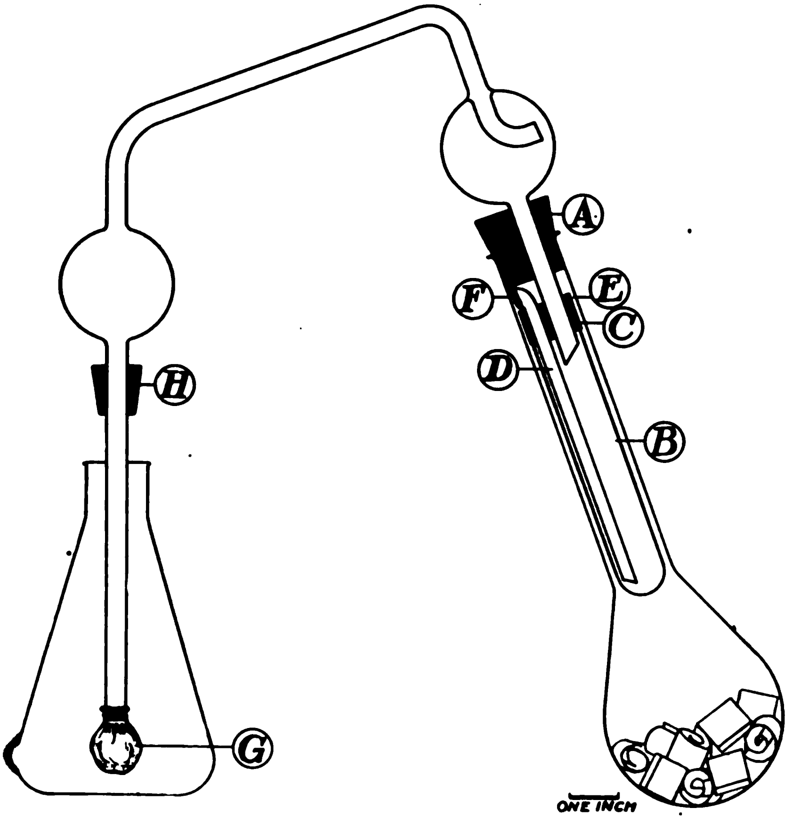

Fig. 1. Apparatus for the determination of nitric nitrogen with the zinc-copper couple.

stopper is covered with half inch electric tape which is wound twice around. This part of the apparatus should be made so that the sides of the test-tube will be a uniform distance of 2 or 3 mm. from the neck of the distilling flask.

The receiving end (F) of the glass tube (D) may be made by sealing the end of the tube and then blowing a large hole in the side just below the seal.

The glass perforated spherical enlargements on the delivery end of distillation tubes insure better absorption in the acid solution than a plain tube but a still more efficient bulb for this purpose is easily made of cambric[5] by cutting out a circle of the cloth 9 cm. in diameter and then gathering it in folds around the delivery end of the tube (G), and tying it with a cotton string which like the cloth has previously been boiled in distilled water. The bag thus formed should measure, when pulled down, about 3.5 cm. from the bottom up to the string. All the cloth above the string should be cut down close to it. The entire surface of this bag in the receiving solution being acid, much of the ammonia is neutralized before it is forced through the cloth. The steam comes through the surface of the cloth in a uniformly fine spray till the receiving solution becomes hot enough when the size of the bubbles increase, but under ordinary conditions they do not exceed 1 mm. in diameter. Bags thus prepared will last a long time. Those used in this work have been in at least 500 determinations.

The writer usually places a rubber stopper (H) just under the glass bulb to facilitate raising the tube out of the receiving solution at the end of the distillation.

Solutions and Reagents.

Indicator.—A test of various indicators showed that *p*-nitrophenol is the most satisfactory one for this work as it gives a sharp end-point in solutions ranging in temperature from 37—70°C. The solutions at the lower temperature stood in cold water for a short time while those at the higher temperature were titrated 5 minutes after being removed from the distilling shelf. The indicator is prepared by dissolving 1 gm. of the commercially pure substance in 100 cc. of 50 per cent alcohol.

Alkaline Solution.—The $\frac{N}{14}$ alkaline solution was prepared from Merck's sticks of potassium hydroxide purified by alcohol, and standardized against chemically pure benzoic acid.[6]

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[5] Cambric 100 (Berkeley Mills). The weight of 100 sq. cm. of this material unboiled is 0.8 gm.

[6] The c.p. benzoic acid was obtained from the Bureau of Standards. Their directions were followed with regard to the standardization.

Acid Solution.—The sulfuric acid solution was made exactly equal in strength to the potassium hydroxide solution.

Ammonia-Free Water.—The water used for all this work was redistilled after acidifying with sulfuric acid to remove ammonia.

Sodium Nitrate Solution.—The sodium nitrate standard was prepared by neutralizing titrated nitric acid solution with sodium hydroxide.

Zinc.—The zinc used for the couples is ordinary sheet zinc about 0.5 mm. thick. It is cut in strips 2 cm. wide by 8 cm. long. These strips are coiled by rolling around a pencil and then opened so that the outermost circle of the coil is approximately 2 cm. in diameter. A coil weighs about 5 gm. 80 gm., or sixteen of these coils, are dropped into each of the flasks. There ought to be about 200 cc. of water in the flask so that the coils will not directly strike the glass as they fall in. The coils may be left in the flasks and used indefinitely if their weight is maintained at 80 gm. by adding one new coil about every week when they are used continually. The flask holders of Pickel[7] are very useful for the flasks containing the coils. In using zinc for the first time it should be treated with an approximately 1 per cent sodium hydroxide solution and then with a 1 per cent sulfuric acid solution for 3 or 4 minutes till it has been well cleaned and a fresh surface of the metal is exposed. The acid solution is then drained from the flask and the couple is ready to be treated with the acid copper sulfate solution.

Acid Copper Sulfate.—This solution is prepared by dissolving 10 gm. of $CuSO_4 . 5H_2O$ in 2 liters of distilled water and then adding 6 cc. of concentrated sulfuric acid.

Alumina Cream.—This suspension is made by dissolving 50 gm. of $Al_2(SO_4)_3 . 18H_2O$ in 2 liters of distilled water, and then adding a few drops of some indicator and neutralizing with an approximately normal sodium hydroxide solution. The solution is well stirred and the precipitate allowed to settle, when the supernatant liquid may be siphoned off and more distilled water added. The precipitate is then allowed to settle over night and the supernatant liquid again siphoned off. The thick cream is kept for clarifying soil extracts.

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[7] Pickel, J. M., *J. Ind. and Eng. Chem.*, 1915, vii, 787.

Magnesia.—The Baker and Adamson Chemical Company's heavy magnesium oxide c.p. was used for all determinations.

Sodium Chloride.—A c.p. salt was used for the electrolyte.

PROCEDURE.

The procedure for making a determination is simple and will lend itself readily to routine work. 150 cc. of the acid copper sulfate solution are poured into the flask containing the zinc and allowed to stand at least 8 minutes. The solution may be left in contact with the zinc while the soil extracts are being filtered. It is then poured off and the couple washed once with water. Tap water may be used if it is all poured out again.

The soil extract is prepared by adding 300 cc. of ammonia-free water to 100 gm. of air-dry soil, or if the soil is high in organic matter 25 cc. of alumina cream and 275 cc. of water. This is allowed to stand half an hour with occasional shaking and is then filtered. 200 cc. of the soil extract is poured into the flask and a measureful of a mixture of 5 gm. of sodium chloride and 1 gm. of magnesia emptied into it. The rubber stopper of the connection tube is fitted into the mouth of the flask, and the delivery end of the connecting tube is placed in the receiving flask, which contains the $\frac{N}{14}$ acid and sufficient distilled water to bring the volume to 100 cc. The gas is lighted and the flame adjusted so that the contents of the flask will boil in 9 to 12 minutes. The boiling is continued for 45 minutes, during which time at least 125 cc. of the soil extract should be distilled over. The receiving flask is then lowered and the cambric bag washed with only 1 or 2 cc. of distilled water, so that no vacuum will be formed in the connection tube. When the tube has been slightly cooled by this small addition of water more may be thrown onto the bag where it is tied to the glass tube. This is the only part that it is necessary to wash as the steam will clean out the bag proper. The distillate is allowed to stand about 5 minutes after the distillation is finished so that the temperature will be between 3 and 70°C., when five drops of the *p*-nitrophenol are added and the titration is made with the $\frac{N}{14}$ caustic potash.

It sometimes happens that when the distillation is run too slowly the scrubbing tube becomes almost filled with water

This condition may be remedied by turning out the gas and as soon as a little of the receiving solution has been drawn up into the connecting tube to form a seal, lowering the receiving flask. The vacuum produced by the cooling of the distillation flask will soon cause the contents of the scrubbing tube to be siphoned over. The gas may then be lighted and the distillation started again.

DATA.

As high organic matter in the solution containing nitrate generally interferes with the accuracy of the determination when made by direct reduction methods, it seemed desirable to find some simple way of removing the bulk of soluble organic matter, so as to eliminate as far as possible any interference by this material with the free generation of hydrogen.

After trying a number of substances it was found that alumina cream would clarify the extract and remove sufficient organic matter to permit a perfect working of the method.

To test the method under rather extreme conditions an extract was prepared from rotted manure by adding 500 cc. of distilled water to 50 gm. of the well dried material. The suspension was frequently shaken and after standing 2 hours was filtered.

15 cc. of this very dark brown solution were added to each of five carefully prepared 100 gm. samples of air-dry greenhouse soil. 25 cc. of alumina cream were added to each sample and in addition 260 cc. of distilled water. Each sample was well shaken and after standing half an hour was poured onto a filter; the first runnings were poured back and an almost perfectly clear and colorless filtrate was obtained. 200 cc. of each of the filtrates were poured into Kjeldahl flasks, 1 gm. of magnesia was added, and the extract boiled to half its volume to expel ammonia. When this was completed the solution, after standing a short time, was cooled by the addition of 50 cc. of redistilled water and then poured onto the couple, the flask was rinsed with approximately 35 cc. of distilled water and then 14.55 mg. of nitric nitrogen were added. The nitrate was not added till just before beginning the distillation in order to eliminate any errors that might arise from manipulation since the test was made to determine whether distillation with these reagents would yield accurate results in the presence of organic matter.

5 gm. of sodium chloride and about 0.5 gm. of magnesia were added to each solution. The extra amount of magnesia was put in to make up for a small quantity lost in transferring the solutions. The flasks were then connected with the rest of the apparatus and the distillation was started. The receiving flasks for the distillates from the soil and manure extracts

contained 25 cc. of standard acid and those for the distillates from the extracts with added nitrate 45 cc. of acid.

A second set of determinations, Nos. 6 to 10, were made with a slightly stronger manure extract and a larger quantity of added nitrate. The titration of the distillates gave the following results.

TABLE I.

Nitric Nitrogen Recovered from Soil and Manure Extract.

Source of extract.	Nitric nitrogen.			
	Added.	Theoretical total.	Found.	Average.
	mg.	*mg.*	*mg.*	*mg.*
1. Soil and manure..............	0.0	10.85	10.9	
2. " " "	0.0	10.85	10.8	10.85
3. " " "	14.55	25.40	25.15	
4. " " "	14.55	25.40	25.35	
5. " " "	14.55	25.40	25.45	25.31
6. " " "	0.0	11.93	12.00	
7. " " "	0.0	11.93	11.85	11.93
8. " " "	15.2	27.13	26.95	
9. " " "	15.2	27.13	27.05	
10. " " "	15.2	27.13	27.15	27.05

The superiority of this method of distilling from a solution made alkaline with only 1 gm. of magnesia is readily apparent.

Allen,[8] appreciating its advantage says: "A very large amount of effort was spent in this work in an attempt to obtain a reliable and accurate method by the reduction in the presence of MgO and distillation with an ordinary Kjeldahl rack, a method which would be extremely simple and rapid."

The low alkalinity produced by this quantity of oxide practically eliminates any possibility of breaking down organic material in the soil extracts.

To test the action of this alkali on organic material duplicate determinations were run with distilled water to which had been added 10 mg. of nitric nitrogen and approximately 10 mg. of nitrogen as urea, sodium asparaginate, glycocoll, peptone, or dried blood. The averages of the duplicate determinations are given in Table II.

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[8] Allen, E. R., *J. Ind. and Eng. Chem.*, 1915, vii, 521.

TABLE II.

Nitric Nitrogen Recovered without Hydrolysis of Organic Substances.

Substance.	Organic nitrogen. Quantity.		Nitrogen.		
	Material.	Nitrogen.	Nitric quantity.	Total.	Recovered.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Urea	21	9.79	10.0	19.79	9.95
Sodium asparaginate	122	9.88	10.0	19.88	9.93
Glycocoll	54	10.04	10.0	20.04	9.94
Peptone	66	9.78	10.0	19.78	9.92
Dried blood	91	12.79	10.0	22.79	9.99

A determination made with a solution containing only urea yielded only the small quantity of ammonia contained in the distilled water.

Probable Error.

As the results obtained with this method were accurate it seemed desirable to ascertain the probable error occurring under

TABLE III.

The Probable Error of a Nitric Nitrogen Determination.

	Nitric nitrogen recovered.	
Theoretical value	3.00	25.00
Experiment No.	*mg.*	*mg.*
1	3.00	24.90
2	2.98	24.90
3	3.01	25.00
4	2.98	24.80
5	3.03	24.90
6	3.00	25.00
7	2.95	24.80
8	3.00	25.10
9	3.02	24.80
10	2.97	24.92
11	2.97	24.95
12	2.90	24.96
Average	2.98	24.92
Deviation from theoretical value	−0.02	−0.08
Probable error	±0.007	±0.044

the regular procedure. Freshly prepared couples were accordingly made for twelve determinations with 3 mg. and twelve with 25 mg. of nitric nitrogen and the probable error of a single determination calculated from the results obtained by the method of least squares.

The low probable error shows that the method may be employed where the most accurate results are desired.

DISCUSSION.

The author has found that some couples will yield good results without renewing them for four distillations when there is as much as 25 mg. of nitric nitrogen to be reduced. Such results depend on the age of the zinc, the quality of the couple formed, and possibly other factors. Because of the complexity of this condition the author does not recommend the repeated use of a couple where more than 5 mg. of nitric nitrogen are to be determined and even then if it is a good couple it should not be used for more than four determinations. For the present, until some method is found either for improving or for estimating the condition of a couple, it should be renewed before each determination. When the couple is to be employed without recoppering it should be washed once and used as soon as possible after distillation is finished. The couple should not be left standing in the residue of a distillation over night for the magnesia cakes around the zinc which is then very difficult to clean even by the acid treatment. If the zinc is washed shortly after a distillation a good couple can be made simply by adding the required amount of acid copper sulfate solution. It is, however, well to treat the zinc with 1 per cent sulfuric acid solution every 3 or 4 days to insure the formation of a good couple.

If the solution containing the nitrate is strongly alkaline it should be acidified with sulfuric acid and an excess of magnesia added because strong alkalinity interferes with the generation of hydrogen by this couple.

SUMMARY.

1. A new method for the determination of nitric nitrogen is described. A zinc-copper couple will reduce a nitrate in solution

boiling point; therefore the reduction proceeds during the ation. 2. A simple apparatus has been made to give excel- esults with this method. 3. Accurate results are obtained solutions high in organic matter if they are clarified with all quantity of alumina cream. 4. Unstable organic matter broken down by this procedure, owing to the slight alka- produced by 1 gm. of magnesia. 5. The probable error that the method is accurate.

THE MECHANISM OF THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

I. THE NECESSITY OF A GENERAL SALT EFFECT UPON THE MEMBRANE AS A PREREQUISITE FOR THIS DIFFUSION.

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INTRODUCTION.

In this series of publications the writer intends to prove the existence of what he believes to be a new principle in the mechanism of the diffusion of certain electrolytes through the membranes of living organs or cells; namely, that this diffusion depends, in addition to the osmotic pressure of the electrolyte, upon a second effect which he calls the general "salt effect" upon the membrane (or certain constituents of the membrane).[1] Last year Loeb and Cattell published the observation[2] that when eggs of *Fundulus* were washed for 24 hours in distilled water and then transferred to a solution of a potassium salt, it took a longer time before the hearts of the embryos inside the egg stopped beating than when the eggs were transferred directly from sea water into the same solution of the potassium salt. This result was ascribed by us to the fact that in the distilled water the eggs lose the salts which are in chemical combination with the surface or the superficial layer of the membrane, and that this lack of salts at the surface was the reason for the retardation of the diffusion of the potassium salts into the egg. This view was supported by the measurements of the osmotic pressure of the juice pressed out from washed and unwashed eggs,[3] the osmotic pressure of the latter being a trifle higher. It is our intention to show in this paper that the diffusion of KCl into washed eggs can be accelerated by adding to the KCl solution a moderate amount of salt; *e.g.*, sea water or a mixture of NaCl and $CaCl_2$ or NaCl alone, thus proving that the retardation of the diffusion of potassium salts into washed eggs is due to the lack of salts at the surface of the egg membrane. The presence of these salts at the external surface of the egg membrane furnishes that second factor—the salt effect—required for the diffusion of the potassium salts. When washed eggs are put into a pure solution of KCl the diffusion of the latter salt into the egg (*i.e.*, through the external surface or layer of the membrane) is delayed until so much KCl has combined with certain elements of the surface of the membrane as to supply the "salt effect."

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[1] Loeb, J., *Proc. Nat. Acad. Sc.*, 1916, ii, 511.

[2] Loeb, *Proc. Nat. Acad. Sc.*, 1915, i, 473. Loeb, J., and Cattell, McK., *J. Biol. Chem.*, 1915, xxiii, 41.

[3] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1915, xxiii, 157.

Before entering into a description of the experiments it will be well to prove that we are dealing in these experiments with an action upon the membrane of the egg and not upon the embryo or upon its heart. It is one of the shortcomings of the usual experiments on salt action that we are at a loss to decide whether we are dealing with a salt action on the membrane or on the adjacent protoplasm. Thus when we find that $CaCl_2$ inhibits the twitching of muscles or nerves caused by Na_2SO_4, Na_3 citrate, or $N(C_2H_5)_4Cl$, we may be in doubt whether this is due to a prevention of the diffusion through the membrane or to an action of Ca upon the living protoplasm, though the evidence speaks in favor of the former possibility.[4] In the experiments on the eggs of *Fundulus* we are relieved of this difficulty since we can always compare the action of the salt on the egg with its action upon the embryo just hatched and thus freed from the protection of the egg membrane. Such a comparison gives us the certainty that in our experiments we are dealing with the effects of salts upon the egg membrane which we must conceive as impermeable for water and nearly impermeable for salts.

We shall see in this paper that an M/8 solution of KCl causes cessation of the heart beat of the embryo in the washed egg within a certain time, that the addition of a definite but small quantity of the salts of sea water or of NaCl and $CaCl_2$ accelerates the poisoning, and that a slightly higher concentration of the same salts inhibits the poisoning. That all these phenomena are due to an action on the membrane of the egg is proved by the fact that two salts utterly incompatible with the life of the embryo or with heart action, *e.g.*, $NaNO_3 + MnCl_2$, will produce the same effect as $NaCl + CaCl_2$. This would be impossible of explanation on any other basis than that $NaCl + CaCl_2$ as well as $NaNO_3 + MnCl_2$ produce the salt effect as well as the antagonistic effect by acting on the membrane of the egg and not by acting on the heart or the embryo.

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[4] Loeb, J., and Ewald, W. F., *J. Biol. Chem.*, 1916, xxv, 377.

In 1905[5] the writer expressed the idea that the antagonistic action of traces of a salt with bivalent cation to high concentrations of a salt with univalent cation in the egg of *Fundulus* was due to a prevention of the diffusion of the latter salt through the membrane; and this conclusion was based on the following fact. When newly fertilized eggs are put into a 5/8 M NaCl solution no egg can form an embryo, while the addition of a trace of $ZnSO_4$, lead acetate, $MnCl_2$, or practically of any salt with a bivalent cation, allowed all or many of the eggs to form embryos. When, however, the newly hatched fish is put into a 5/8 M NaCl solution it dies even more quickly if a trace of $ZnSO_4$ or lead acetate or $MnCl_2$ is added than in the pure 5/8 M NaCl solution. For the embryo outside the membrane of the egg only Ca or Sr or Mg can be used as antagonists to higher concentrations of NaCl. This shows that the prolongation of the life of the egg in a 5/8 M NaCl solution through the addition of a bivalent cation is due to a modification of the membrane by the latter whereby the membrane is rendered impermeable for these salts.

Moreover when we put the eggs of *Fundulus* with beating hearts into a pure 3 M solution of NaCl the eggs are killed in about 3 hours while they live for 3 to 5 days when we add the proper amount of Ca.[6] We can prove with certainty that the Ca acts by preventing the diffusion of the NaCl into the egg, since if we put the newly hatched embryos into the same 3 M $NaCl + CaCl_2$ solution in which the unhatched embryo of the same age lives 3 to 5 days, they die in less than 10 minutes. The young larvæ of the fish freed from the membrane cannot live in a solution of NaCl + $CaCl_2$ higher than 10/8 M.[7] Hence we may be certain that only traces of a solution of 3 M NaCl + $CaCl_2$ diffuse through the membrane of the eggs so that it takes from 3 to 5 days to allow so much NaCl + $CaCl_2$ to diffuse through the membrane as is required to raise the concentration inside the membrane to 10/8 M or above.

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[5] Loeb, *Arch. ges. Physiol.*, 1905, cvii, 252.

[6] Loeb, *Science*, 1912, xxxvi, 637; *Biochem. Z.*, 1912, xlvii, 127.

This statement can be corroborated by determinations of the specific gravity of fertilized eggs of *Fundulus*. The method of these experiments consisted in ascertaining the concentration of NaCl solution in which the eggs were just able to float. The normal egg of *Fundulus* has a specific gravity higher than sea water but less than that of a 14/8 M NaCl solution.[8] Thus it was ascertained that eggs kept for 6 days in sea water floated in 14/8 M and sometimes in 13/8 M NaCl; eggs of the same female kept 6 days in distilled water floated in 13/8 M NaCl and sometimes in 12/8 M NaCl; eggs kept for 3 days in 3 M NaCl + $CaCl_2$ sank at first in a 15/8 M NaCl solution but rose again in a few minutes and remained at the surface (after having lost the salt solution which adhered at their surface when first brought into the 15/8 M NaCl from the 3 M NaCl + $CaCl_2$ solution). They were not able to float in a 14/8 M NaCl solution. This experiment gives an indication of how slowly a balanced solution or H_2O diffuses into or through the membrane. In a 3 M NaCl + $CaCl_2$ solution the specific gravity of eggs increased in 3 days only about 7 per cent and in H_2O the specific gravity diminished to about the same amount in 6 days.

Since from our experiments we drew conclusions upon the diffusion of KCl through the membrane of the egg it may be well to show the difference of the action of KCl solutions upon the embryo when inside the membrane and when freed from the membrane. The embryo inside the egg can live more than a week in a pure M/16 KCl solution, especially when the egg is previously washed in H_2O or in dilute sea water; *e.g.*, M/80. The fish themselves succumb to a pure M/16 KCl solution in less than 1 hour, no matter whether they are put directly into the M/16 KCl solution from normal (M/2) sea water or whether they are previously kept for 6 days in M/80 sea water. In the newly hatched fish the heart stopped beating in an M/8 KCl solution in considerably less than 30 minutes, often in 10 minutes, while the embryos of the same lot of eggs but still inside the membrane were not poisoned by the same KCl solution in 24 hours, and if the eggs were previously washed in H_2O or M/80 sea water they could live in such a solution as long as 6 days. This proves that H_2O as well as the salts penetrate the membrane extremely slowly and that the salt effect as well as the antagonistic salt action to be described in this paper are due to modifications of the egg membrane and not to an action upon the embryo itself.

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[7] Loeb, *Biochem. Z.*, 1913, liii, 391.

[8] Loeb, *Biochem. Z.*, 1912, xlvii, 127.

In our experiments we generally only modify the salt contents of the external surface or layer of the egg membrane. The internal layer is in contact with the liquid of the egg, *e.g.*, a solution with a freezing point depression of between 0.75 and 0.77°.[3] It is probable that the osmotic pressure of the liquid inside the egg is furnished chiefly by electrolytes. This liquid is generally not affected by the washing of the eggs.

I. The Difference in the Rate of Diffusion of Potassium Salts into Washed and Unwashed Eggs.

Eggs washed for 24 hours in distilled water and unwashed eggs (taken directly from sea water) were put into different concentrations of a potassium salt, *e.g.*, KCl or K_2SO_4, and after definite intervals the rate of poisoning in the two sets of eggs was ascertained by counting the percentage of hearts still beating in each solution. The smaller this percentage at a given time, the greater the rate of poisoning. Each solution contained about twenty eggs (about 1 week old and with heart beat and circulation well established). Tables I and II show the difference in the rate of poisoning between the washed and unwashed eggs. This difference corresponds exactly with our theory. The *unwashed* eggs (Table I) contain at the beginning enough salt at the surface of their membrane to permit the rapid diffusion of K_2SO_4 into the egg. Accordingly we notice that in all solutions a sharp drop in the percentage of beating hearts occurs during the first 2 or 3 hours, during which time the surface of the membrane has still enough salt for the salt effect. After this period the further drop in the percentage of heart

TABLE I.

After	Percentage of unwashed eggs (taken directly from sea water) with beating hearts in K_2SO_4.							
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256
hrs.								
½	80	85	100	100	100	100	100	100
1	50	75	80	80	75	75	95	100
2	0	30	50	50	65	50	95	95
5		20	45	45	45	50	80	80
11		10	40	40	40	50	65	70
22		10	35	40	55	55	70	70
48		0	15	40	45	50	70	70
78			10	35	40	55	65	70
123			5	25	30	45	60	75
175			0	35	30	20	45	75
267				0	5	0	5	15
343					0	0	0	0

beats is slow, for the reason that by this time the greater amount of salts of the sea water has diffused from the surface of the membrane into the outside solutions. From now on the further diffusion of K_2SO_4 into the egg is retarded until enough of this salt has entered into combination with the outer surface (or layer) of the membrane to supply the general salt effect.

On the other hand, the washed eggs (Table II) contain at the beginning no or only an inadequate amount of salt at the surface of their membrane, and hence according to our theory the diffusion of K_2SO_4 through the membrane should be impossible. A glance at Table II shows that this is the case, inasmuch as during the first 2 hours not a single heart stops beating in the solutions below M/2 K_2SO_4 and during the first 12 hours none stop beating in the solutions below M/8 K_2SO_4. The diffusion of K_2SO_4 into the washed eggs can only begin after enough K_2SO_4 has combined with the outer surface or layer of the membrane to furnish the general salt effect required for the diffusion of the potassium salts through the membrane. This happens the more rapidly the higher the concentration of the K_2SO_4 solutions. After once the surface of the membrane is supplied with the necessary amount of salt for the salt effect, the hearts stop beating rather rapidly.

TABLE II.

After	Percentage of washed eggs with beating hearts; *i. e.*, of eggs kept for 24 hrs. in distilled water before being put into K_2SO_4.							
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256
hrs.								
1	90	100	100	100	100	100	100	100
2	35	100	100	100	100	100	100	100
4	0	75	100	100	100	100	100	100
12		50	100	100	100	100	100	100
23		5	85	100	100	100	100	100
47		0	80	100	95	85	100	100
78		0	80	100	90	80	95	100
123			40	92	90	50	85	95
177			15	70	50	45	55	75
268			5	5	10	0	5	15
343			0	0	0	0	0	0

It may perhaps be desirable to follow these results a little more in detail. We begin with the M/8 K_2SO_4 solution.

Half of the *unwashed* eggs stop beating in an M/8 K_2SO_4 solution in 2 hours (Table I), while not a single one of the *washed* eggs shows a cessation of the heart beat in the same time in an M/8 K_2SO_4 solution (Table II), and only a slight poisoning effect appears in the washed eggs in an M/8 K_2SO_4 solution until the 4th day. From that time on a more rapid increase in the percentage of poisoned hearts is found in the washed eggs owing to the gradual production of a salt effect upon the membranes by the M/8 K_2SO_4 solution itself. In the *unwashed* eggs there is a rapid drop in the percentage of beating hearts during the first few hours (Table I); after this time the number of eggs with beating hearts remains almost constant, since the salts have diffused away from the surface of the eggs into the outside solutions. From now on the further salt effect upon the membrane has to be supplied by the M/8 KCl solution itself. This is a slow process and we therefore notice a very slow increase in the percentage of poisoned eggs after the first 6 hours in the unwashed eggs (Table I). The same difference exists for the M/16, M/32, and M/64 K_2SO_4 solution. Half of the *unwashed* eggs stop beating in these solutions in the first 3 hours, owing to the presence of the salts of the sea water at the surface of the membrane; while it requires a week or more before the same effect is produced in the *washed* eggs by the same solutions.

In the M/4 solution, 70 per cent of the unwashed eggs had stopped beating in the first 2 hours (Table I), while not a single one of the washed eggs (Table II) was poisoned in this time, owing to the lack of salts at the surface of the latter and of their presence at the surface of the unwashed eggs. After this time the M/4 K_2SO_4 is beginning to produce the salt effect and now the washed eggs are poisoned almost as rapidly as the unwashed eggs. This is shown also in the fact that it requires about as much time to poison the last egg of the unwashed as of the washed eggs in the M/4 K_2SO_4 and the weaker solutions. In the M/2 K_2SO_4, the difference between the washed and unwashed eggs becomes less marked simply because the M/2 K_2SO_4 solution can produce the salt effect upon the membrane of the washed eggs more rapidly.

Similar observations as in the case of K_2SO_4 were made in regard to the effect of KCl solutions upon washed and unwashed eggs. Thus more than 50 per cent of the hearts of unwashed eggs ceased to beat in an M/8 KCl solution after less than 1½ hours while 50 per cent of the hearts in the washed eggs were still beating after 2 days in the same solution.

These experiments therefore harmonize with the idea that the rapidity of the diffusion of a potassium salt through the membrane of the egg of *Fundulus* depends, in addition to the osmotic pressure of the KCl solution, upon the presence or absence of salt at the surface or the superficial layers of this membrane.

II. The Acceleration of the Diffusion of KCl through the Addition of a Moderate Amount of a Different Salt, and the Retardation of this Diffusion by the Addition of a Greater Amount of Salt.

We can prove the correctness of the last statement by putting washed eggs of *Fundulus* (*a*) into pure M/8 KCl solutions and (*b*) into M/8 KCl solutions to which some sea water or some other salt solution has been added. If it is true that the hearts of the washed eggs in the previous experiments stopped so much later because their membranes did not contain enough salt, the addition of some sea water or some other salt solution should accelerate the poisoning of washed eggs in an M/8 KCl solution; and the more so the higher (up to a certain limit) the concentration of the sea water. Eggs were put for 24 hours in H_2O (after having been washed three times in H_2O) and were then distributed in M/8 KCl solutions made up in H_2O and in increasing concentrations of sea water up to normal sea water. We call the concentration of our sea water M/2 although it is a trifle higher, and we call twice diluted sea water M/4, and so on. Table III gives the result of the experiment.

TABLE III.

After	Percentage of washed eggs with beating hearts in M/8 KCl made up in sea water or H_2O.								
	Sea water.								H_2O
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	
days									
1	24	70	46	65	63	72	85	82	96
2	10	20	30	44	55	65	55	63	80
3	35	0	16	34	28	42	55	50	55
4	30	0	8	8	14	36	20	37	45
5	10	0	4	0	9	27	15	25	25
6	0	0	0	0	9	18	15	18	15
7	0	0	0	0	9	13	10	6	0

While it was more than 3 days until 50 per cent of the hearts of the washed eggs stopped beating when put into a pure M/8 KCl solution, in an M/8 KCl solution in undiluted sea water it took considerably less than a day, and in an M/8 KCl solution made up in twice diluted (M/4), four times diluted (M/8), and eight times diluted (M/16) sea water it took less than 2 days. In M/8 KCl dissolved in M/128 and M/256 sea water the eggs behaved as they did in the pure M/8 KCl solutions. This proves that our contention was correct that the difference between the rate at which washed and unwashed eggs were poisoned by the solution of potassium salt was due to lack of salts on the surface (or the superficial layer) of the membrane. Another interesting fact appears in these experiments. M/4 sea water, while less efficient during the first 2 days of the experiment than undiluted sea water, becomes more efficient from the 3rd day on;

because, as we assume, the amount of salts which diffuses into the surface layer of the membranes in the undiluted sea water exceeds the quantity required for the acceleration of the diffusion of KCl and the opposite effect is produced; namely, a retardation (antagonistic salt action).

Since the sea water contains a small amount of KCl it was necessary to show that the substitution of a physiologically balanced salt solution (free from KCl) for sea water gives the same result. Such a solution affords us also an opportunity to prove *that while the addition of a moderate amount of salt (e.g.,* M/8 *or* M/4) *accelerates the rate of diffusion of* M/8 *KCl into the washed eggs, a slightly higher concentration of NaCl + $CaCl_2$,* M/2 *or* M/1, *has the opposite effect; namely, to retard or prevent the diffusion of KCl into the egg,* with the exception of the first hour or more, when they also accelerate the diffusion.

Eggs washed for 24 hours in H_2O were distributed into M/8 KCl solutions made up in H_2O and in different concentrations of NaCl + $CaCl_2$ (in the proportion in which these salts are contained in sea water, 100 molecules of NaCl to 1.75 molecules of $CaCl_2$).

TABLE IV.

After	Percentage of washed eggs with beating hearts in M/8 KCl in NaCl + $CaCl_2$ or H_2O.									
	NaCl + $CaCl_2$									H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	
days										
1	100	73	74	90	95	96	87	75	85	96
2	100	77	38	64	85	84	66	40	85	80
3	100	77	9	20	40	25	53	20	85	55
4	100	73	0	0	25	10	50	20	80	45
5	100	67	0	0	20	10	47	20	70	25

We notice the same fact as in the experiment with sea water; namely, that if an M/8 KCl solution is made up in M/4 or M/8 solution of NaCl + $CaCl_2$ the velocity with which KCl diffuses into the egg is accelerated, thus proving that the action of sea water was not due to the KCl it contained. Thus after 3 days, more than 50 per cent of the eggs had still beating hearts in the ure M/8 KCl solutions, while in the M/8 KCl solution made up n M/4 NaCl + $CaCl_2$ only 9 per cent had beating hearts. After days all the hearts had stopped beating in M/8 KCl solutions ade up in M/4 and M/8 NaCl + $CaCl_2$ while in the pure M/8 Cl solutions still 45 per cent of the eggs had beating hearts.

The M/8 KCl solution made up in M/2 NaCl + $CaCl_2$ is of pecial interest. During the 1st day (and very probably durg the first hours of the 1st day) the number of eggs with beatg hearts fell to 73 per cent while in the pure M/8 KCl solution fell only to 96 per cent. Therefore the M/2 NaCl + $CaCl_2$ t first accelerates the diffusion of KCl through the membrane. his is quite intelligible on the basis of our theory. The equilibium between the surface of the membrane and the salt soluion was not established at once but only gradually, and before he equilibrium was complete there existed a condition in which he membrane had as much salt as corresponded to the equilibium with an M/8 and later a M/4 solution of NaCl + $CaCl_2$ which ccelerate the diffusion. During this period 27 per cent of the ggs were poisoned. Then more salt entered into combination ith the membrane and the opposite result was produced; namely, n impermeability to M/8 KCl. We therefore see that during he next 4 days no more eggs were poisoned with KCl. In the /1 NaCl + $CaCl_2$ this latter condition was produced so rapidly hat there was no chance for the diffusion of enough KCl into he eggs to cause the cessation of heart beats. All the hearts ere still beating in one experiment in an M/8 KCl solution ade up in M/1 NaCl + $CaCl_2$ + $MgCl_2$ + $MgSO_4$ after 19 ays, when the experiment was discontinued.

The preceding experiments have shown that the acceleration f the rate of poisoning with M/8 KCl in M/8 or M/4 sea water due to a salt action upon the membrane. We can also show hat the prevention of the poisoning by an M/8 KCl solution hrough a M/1 or M/2 solution of NaCl + $CaCl_2$ is not due to n action of these latter salts upon the heart, but to an action pon the membrane. This proof is furnished by the fact already entioned in the introduction, that a solution utterly incomatible with the activity of the heart of *Fundulus*, *e.g.*, $NaNO_3$ + nCl_2, acts in the same way as NaCl + $CaCl_2$, as shown in able V. Eggs washed 24 hours in H_2O were put into M/8 KCl made up in H_2O and different concentrations of $NaNO_3$ + $MnCl_2$ (100 molecules of the $NaNO_3$ to 1.75 molecules of the $MnCl_2$).

TABLE V.

After	Percentage of washed eggs with beating hearts in M/8 KCl in $NaNO_3$ + $MnCl_2$ or H_2O.							
	$NaNO_3$ + $MnCl_2$							H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	
days								
1	100	45	44	44	90	85	90	85
2	100	45	40	58	85	75	75	60
3	100	45	30	50	75	60	50	50
4	90	45	10	20	45	65	50	40
6	70	34	0	0	33	30	30	10

This experiment shows that the M/8 KCl solution was not able to poison any of the eggs when the solution was made up in a M/1 solution of $NaNO_3$ + $MnCl_2$. This protective or antagonistic action of $NaNO_3$ + $MnCl_2$ could only have been due to a salt effect upon the membrane, whereby the diffusion was prevented. While in the pure M/8 KCl solution 85 per cent of the embryos were still alive after 1 day, in the M/8 KCl solution made up in M/2, M/4, and M/8 solution of $NaNO_3$ + $MnCl_2$ more than half of the hearts of the embryos had stopped beating in the same time; while in the M/8 KCl made up in M/16, M/32, and M/64 solution of $NaNO_3$ + $MnCl_2$ the embryos behaved like those in the pure M/8 KCl. We notice again the characteristic phenomenon that in M/8 KCl made up in M/2 $NaNO_3$ + $MnCl_2$ during the 1st day a sharp drop occurs in the number of beating hearts (to 45 per cent), while in the pure M/8 KCl solution at this time only 15 per cent of the hearts have stopped beating. After that the number of eggs with beating hearts remains constant in the M/8 KCl solution made up in M/2 $NaNO_3$ + $MnCl_2$ while in the pure M/8 KCl solution it diminishes constantly. The explanation is the same as given for NaCl + $CaCl_2$.

Since it might be thought that this antagonistic action was due to the presence of the bivalent cation Ca or Mn in the solution we may state that non-balanced salt solutions act in the same way. The following experiment (Table VI) with NaBr may serve as an example. Eggs washed for 24 hours in H_2O were put into M/8 KCl made up in different concentrations of NaBr.

TABLE VI.

After	Percentage of washed eggs with beating hearts in M/8 KCl in NaBr, H_2O, or sea water.										
	NaBr									H_2O	M/4 Sea water.
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256		
days											
1	100	95	52	47	75	65	70	74	85	95	35
2	92	85	40	40	65	55	60	57	80	75	15

It is obvious that M/1 and M/2 NaBr solutions prevented the diffusion of KCl into the eggs since practically the hearts of all of them continued to beat. In the M/8 KCl solution made up in M/4 and M/8 NaBr the eggs were poisoned more rapidly than in the pure M/8 KCl solution and the same was true for the eggs in the M/8 KCl solutions made up in M/4 sea water. The M/8 KCl solutions made up in M/16 to M/256 NaBr acted almost like the pure M/8 KCl solution.

CONCLUSION.

All these experiments prove that the diffusion of KCl through the membrane of the egg of Fundulus does not depend only on the osmotic pressure of the KCl solution but that in addition a certain modification of the membrane is required (*general salt effect.*) *This modification is produced by the action of salts upon the external surface or layer of the membrane* (presumably upon the proteins of the membrane). When the eggs are in the sea water the salts of the latter supply this general salt action upon the membrane. When the membranes are sufficiently freed from the salts of the sea water by washing them for 24 hours in H_2O and if they are then put into a pure solution of a potassium salt the poisonous effect of the potassium salt is delayed until the potassium salt itself has had time to supply the salt effect upon the membrane required to permit the diffusion of this salt through the membrane. When the concentration of the potassium salt is low its diffusion into the washed eggs of *Fundulus* can be accelerated by adding some other balanced or non-balanced salt solution, which supplies the salt effect upon the membrane.

When the concentration of the salt solution added to the KCl solution is slightly higher than that required for the accelerating salt effect upon the membrane, the opposite phenomenon is observed; namely, the retardation or prevention of the diffusion of the potassium salt. This latter effect, the antagonistic salt action, has thus far alone aroused the attention of the workers, although the general salt effect discussed in this paper was already noticed 6 years ago when we found that the adult fish of *Fundulus* die more rapidly in M/100 to M/66 KCl solutions when 10 molecules of NaCl are added to 1 molecule of KCl, while the opposite effect, namely, an antagonistic action, results when more (17 molecules or more) NaCl is added to the 1 molecule of KCl.[9] The meaning of the fact, *i.e.*, the necessity of a general salt effect for the diffusion of potassium, was at that time not recognized, but we can now explain these observations on the assumption that while the gills or skin of the fish contain enough salt to permit the diffusion of M/100 KCl through the surface a better salt effect is reached when about 10 molecules of NaCl are added to 1 molecule of KCl. The addition of 17 or more molecules of NaCl to 1 molecule of KCl has the opposite effect of retarding the diffusion of KCl (antagonistic salt action). This observation shows that the phenomena described for the membrane of the egg of *Fundulus* are not confined to this object but are more general in character.

It has become customary in physiology to discriminate between diffusion and secretion, the term diffusion being used when the difference in pressure of the diffusing substance on both sides of a membrane is sufficient to account for the direction of the diffusion; while we speak of secretion when this is no longer possible. Some authors imply that in the latter case specifically vital conditions or conditions inseparable from the life of the cell play a rôle. The question whether the exchange of gases in the lungs is a mere process of diffusion or one of secretion has divided physiologists for some time. It is quite possible that the facts published in this paper will help towards lifting the veil of mystery from certain cases of secretion.

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[9] Loeb and Wasteneys, *Biochem. Z.*, 1911, xxxii, 155.

THE MECHANISM OF THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

II. THE DIFFUSION OF KCl OUT OF THE EGG OF FUNDULUS AND THE RELATIVE EFFICIENCY OF DIFFERENT IONS FOR THE SALT EFFECT.

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I. The Impossibility of Recovery from Potassium Poisoning in Solutions of Non-Electrolytes.

We showed in the first part of this series[1] that the diffusion of potassium salts through the membrane of the living egg of *Fundulus* depends, in addition to the osmotic pressure of the solution, on a second effect which we called the general salt effect. Thus if the external surface of the membranes of the eggs is freed from salt by washing for 24 hours in distilled water, the potassium salts cannot diffuse into the egg until the external surface of its membrane has been again supplied with salts either by adding a second salt or by giving the potassium salt in solution time to enter into combination with certain constituents of the membrane and thus to supply the general salt effect.

Loeb and Cattell[2] have already shown that by depriving the external surface of the membrane of salts an obstacle is created also for the diffusion of potassium salts in the opposite direction; namely, from the interior of the egg into the surrounding solution. When eggs of *Fundulus* are poisoned with KCl until all the hearts stop beating, they can recover and the hearts can begin to beat again, as soon as enough KCl has diffused out of the egg to bring the concentration of KCl inside the egg below that limit which is required for the cessation of the heart beat. Loeb and Cattell found that eggs kept in a KCl solution until all the hearts stopped beating could not recover when put subsequently into distilled water or a solution of cane sugar, while they could recover when put into solutions of electrolytes. The same authors have also shown that in this recovery from potassium poisoning the nature of the anion in the outside solution played an important rôle, inasmuch as the relative efficiency of Cl : SO_4 : citrate was approximately as 1 : 4 : 16 (Hardy's rule).

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[1] Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339.

[2] Loeb, J., and Cattell, McK., *J. Biol. Chem.*, 1915, xxiii, 41.

In our new experiments the eggs were put directly from sea water into a M/2 KCl solution where they remained for 12 hours. Since the hearts stopped beating, as a rule, in less than 2 hours in such a solution, each egg contained an amount of KCl considerably in excess of that needed for the cessation of the heart beat. Each egg also probably contained approximately the same amount of KCl. When such eggs are put from the M/2 KCl into distilled water, they contain at first enough salt at their surface to permit the diffusion of KCl through the membrane, and we may therefore expect that in the first few hours a few of the eggs may recover even in distilled water. But after a few hours the external surface will have lost a considerable part of its salts by diffusion into the outside solution and as a consequence this external surface or layer of the membrane will become impermeable for the KCl and from then on no more eggs should be able to recover. This was actually observed for eggs which were put into H_2O after treatment with KCl and we will show that the same is true if eggs poisoned with KCl are put into any solution of a non-electrolyte.

We first give as an illustration the behavior of eggs poisoned for 12 hours in M/2 KCl and then put into glycerol solutions of different concentrations (Table I). By recovered eggs we mean eggs whose hearts beat regularly again. While 75 per cent of the eggs recovered in sea water inside of 6 hours and all recovered in 24 hours, of those put into H_2O or glycerol solutions very few recovered. The important fact is that the small number of recoveries observed in glycerol and H_2O took place during the first 6 hours only, while the external surface of the egg membrane had not yet lost that quantity of salts required for the general

TABLE I.

After	Percentage of poisoned eggs which recovered in glycerol solutions, sea water, or H_2O.										
	Glycerol.									M/2 Sea water.	H_2O
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512		
days											
¼	26	10	10	11	11	0	10	0	15	75	5
1	26	20	15	11	11	0	15	5	15	100	10
2	21	10	15	11	5	0	10	5	20		10
3	10	10	20	11	5	0	5	5	10		5
4	21	10	10	5	5	0	5	5	10		5
5	21	10	5	5	5	0	5	0	5		5
Eggs then transferred to sea water.											
1	89	100	95	100	100	100	95	95	100		

salt effect upon the membrane. After this no further recovery took place in the next 5 days. Then the eggs were transferred into sea water where the salt effect was supplied. In 24 hours practically all the eggs had recovered; the figures are given above. Table II gives a similar experiment with cane sugar.

TABLE II.

After	Percentage of eggs which recovered in cane sugar or H_2O.											
	Cane sugar.											H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	
days												
¼	14	5	14	5	9	5	15	38	11	23	5	0
1	14	10	14	20	14	16	15	38	22	34	10	0
2	14	15	14	15	5	10	15	22	11	34	10	0
3	14	10	9	10	14	10	5	22	11	23	0	0
4	9	10	9	10	14	5	5	22	0	34	0	0
5	9	10	14	10	5	0	0	0	0	29	0	

Again we notice the same striking phenomenon that what little recovery there is in the solutions of cane sugar takes place during the first 6 hours when the salt has not yet diffused in sufficient quantities from the external surface of the membrane. After 5 days the number of recoveries has not increased beyond that which took place during the first 6 hours. The number of recoveries which took place during the first 6 hours was comparatively large, owing to the fact that either the salt content of these eggs was slightly greater than in the glycerol experiment, or the salts diffused away from the surface of the membrane with less rapidity than in the case of other non-electrolytes.

TABLE III.

After	Percentage of eggs which recovered in grape sugar or H_2O.										
	Grape sugar.										H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	
days											
¼	5	15	15	5	0	0	5	5	5	0	10
1	0	15	10	5	0	0	0	5	0	0	15
2	10	15	20	5	0	5	0	5	0	0	10

The eggs were then transferred to sea water. The recovery took place rather rapidly.

1	74	91	85	75	85	75	66	60	65	70	65
2	100	95	95	100	100	95	95	85	85	90	85

Finally we give a third experiment on the recovery of poisoned eggs in grape sugar (Table III). As in the previous experiments with non-electrolytes a small percentage of recoveries occurred in grape sugar during the first 6 hours when not enough salt had diffused away from the surface of the membrane. After the first hours no further recoveries took place because the outer layer and surface did not contain enough salt for this purpose. As soon, however, as the eggs were put into sea water the recovery became very rapid since then the outer surface and layer of the membrane was supplied with salt.

Experiments with other non-electrolytes (or very weak electrolytes) like ethyl and methyl alcohol and urea gave about the same results as those reported. From all these experiments we can draw the following conclusion: *The diffusion of KCl out of the interior of the poisoned Fundulus eggs cannot take place when the external surface or layer of the membrane contains less salt than that required to produce the salt effect upon the membrane.*

II. The Recovery of Eggs in Balanced and Non-Balanced Solutions.

When we put eggs which had been kept for 12 hours in a M/2 KCl solution into a solution of certain electrolytes instead of non-electrolytes, we observe an altogether different phenomenon from that described for solutions of non-electrolytes. An almost complete recovery of all the eggs takes place in the salt solutions above a certain concentration inside of 6 hours and a gradual recovery in the weaker solutions; since in the weaker solutions enough salt must gradually combine with the outer surface of the membrane to produce enough of a salt effect to allow at least the *slow* diffusion of KCl through the membrane. Table IV gives the recovery in different concentrations of sea water of eggs poisoned for 12 hours previously in M/2 KCl.

TABLE IV.

After	Percentage of eggs which recovered in sea water.											
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096
days												
$\frac{1}{4}$	75	55	20	10	5	20	5	20	0	10	10	20
1	100	100	95	60	50	26	16	45	14	10	25	35
2			100	80	75	33	33	45	14	10	25	30
3				100	90	70	40	45	14	14	10	20
4					100	100	70	75	22	14	10	10
5							84	100	55	14	10	10
6							95	95	65	20	20	15
7							100	100	72	30	15	15

This experiment was carried on with the same material and simultaneously with the glycerol experiment (Table I). If we compare the two we notice the salt effect; namely, the quick recovery of a majority of the eggs in M/2 and M/4 solutions of sea water within 6 hours. In the previous experiment on the diffusion of the KCl in the opposite direction, *into* the egg, we noticed also that the M/2 and the M/4 sea water accelerated the diffusion of KCl through the membrane in the first 6 hours. No such acceleration is found in the experiments with the non-electrolytes. Moreover, with each day more and more eggs recover in the weaker concentrations of sea water, while no such influence of the concentration can be found in the case of non-electrolytes.

We shall now show that there is no great difference between the action of a balanced and a non-balanced solution upon the recovery of the heart from KCl poisoning. We compare the percentage of recoveries in a balanced solution, NaCl + $CaCl_2$, and a non-balanced solution, NaCl. Both experiments were made simultaneously. The eggs had been kept for 12 hours in M/2 KCl, as usual.

TABLE V.

After	Percentage of eggs recovering from potassium poisoning in NaCl + $CaCl_2$ or H_2O.										
	NaCl + $CaCl_2$										H_2O
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	
days											
¼	70	85	75	35	5	15	15	15	15	0	20
1	100	95	100	90	55	55	25	25	5	5	20
2		100	100	100	80	75	40	20	10	15	10
3					90	95	60	50	15	5	10
	NaCl										
¼	55	75	80	60	55	45	20	25	10	10	0
1	100	100	100	100	100	90	40	25	15	10	5
2						95	75	35	15	5	5
3						95	80	60	15	10	5

In both solutions more than 50 per cent of the eggs recover in the concentrations above M/4 in less than 6 hours. The pure NaCl solution is more efficient than the mixture of NaCl + $CaCl_2$. In the M/8 and M/16 NaCl solution 60 per cent and 55 per cent of the eggs recover in the first 6 hours while in the M/8 and M/16 solutions of NaCl + $CaCl_2$ only 35 per cent and 5 per cent recover. After 2 days 75 per cent of the hearts have recovered in the M/64 NaCl and only 40 per cent in the M/64 NaCl + $CaCl_2$. This difference is not accidental but constant. The difference is, however, in harmony with our experience with the antagonism between NaCl and $CaCl_2$ if we assume that the $CaCl_2$ makes the NaCl less effective in its action on the membrane and hence less available for the general salt effect; so that a mixture of NaCl + $CaCl_2$ acts like a pure NaCl solution of a lower concentration.

We may consider it as proved that aside from this latter condition balanced solutions like sea water are just as fit to render the diffusion of KCl out of the egg possible as non-balanced solutions, and that it is therefore impossible to ascribe this result to an "injury" to the membrane.

It was of interest to find out whether higher concentrations of NaCl solution or of NaCl + $CaCl_2$ would prevent the diffusion of KCl out of the egg (antagonistic salt action), but this was not the case. Eggs that had been poisoned in M/2 KCl recovered more promptly when put into 3 M, 2½ M, 2 M, and 1½ M NaCl + $CaCl_2$ than when put into M/2, M/4, or M/8 solutions. The probable explanation is that the diffusion of KCl out of the egg took place in these highly concentrated solutions so rapidly that the excess of KCl had already diffused out of the egg before the antagonistic effect of NaCl + $CaCl_2$ could be established. It requires some time until enough molecules of NaCl + $CaCl_2$ combine with the membrane to inhibit the diffusion of KCl. Until this happens the conditions for the diffusion of KCl out of the egg are most favorable so that the eggs recover before the antagonistic salt effect can make itself felt. We shall see in the third part of this paper that if the eggs are first treated for 24 hours with 2 M NaCl + $CaCl_2$ and are then put into a pure M/8 KCl solution, the outer surface of the membrane contains enough NaCl and $CaCl_2$ for the antagonistic salt action and no KCl can diffuse into the egg. The fact that the addition of sea water makes the recovery of the heart possible is in full harmony with the experiments of the first part, that sea water also accelerates the diffusion of low concentrations of KCl into the egg.

The recovery of the egg from the poisoning in the sea water cannot be ascribed to the diffusion of sea water into the egg (which does not occur) since solutions utterly unable to arouse or maintain the heart beat or the life of the embryo facilitate the recovery of the poisoned embryo; *e.g.*, ammonium nitrate and many others.

III. The Influence of Anions and Cations of Different Salts upon the Recovery of Eggs from KCl.

In Table VI we give the percentage of hearts which recovered from KCl poisoning in different solutions of electrolytes. The eggs had previously been 12 hours in a M/2 KCl solution and were then put for 24 hours into the solutions indicated in Table VI.

TABLE VI.

	Percentage of hearts recovering from potassium poisoning in 24 hrs.													
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	H_2O
Sea water		100	100	91	60	50	26	15	45	15	10	25	35	5
NaCl + $CaCl_2$	100	95	100	90	55	55	25	25	5	5				10
NaCl	100	100	100	100	100	90	40	25	15	10				5
NaBr	100	100	100	100	90	75	66	50	35	30	40			5
$NaNO_3$				100	100	70	35	15	10	15	10	0	10	5
Na_2 acetate		100	100	100	100	100	85	35	15	0	5	5	0	5
Na_2SO_4		100	100	100	100	90	93	90	81	38	14	5		
$NaHCO_3$				100	100	100	100	75	35	10	5			
Na_2CO_3									35	30	20	20	5	5
Na_2HPO_4					95	100	90	55	50	25	10	14	5	0
Na_3 citrate									95	60	80	30	20	
NH_4Cl		38	72	89	83	77	41	16	15	15				11
$(NH_4)_2SO_4$			45	75	70	75	65	65	50	31	15	10	30	10
$(NH_4)_3$ citrate									80	68	55	25	14	
$MgCl_2$			100	85	95	75	55	25	25	25	15	10	0	5
$MgSO_4$		100	95	70	95	90	80	45	70	30	10	10	15	

The order of rising efficiency for the anions for producing the salt effect is as follows:

Cl, NO_3 < Br < Acetate < PO_4 (?) CO_3 < SO_4 < Citrate.

In order to ascertain the influence of valency we may select as a standard of comparison that concentration which causes about 70 per cent of eggs to recover in 24 hours. This concentration is, in the case of Na salts for acetate and approximately also for Br, M/64, for Cl and NO_3 between M/32 and M/64. For SO_4 it is M/256 and for CO_3 between M/128 and M/256. For citrate it is M/1,024 but for PO_4 it is too small, between M/64 and M/128. Omitting Na_2HPO_4 from consideration we find the ratio of efficiency for monovalent: bivalent: trivalent anions as 1 : 4 : 16 which is the expression of Hardy's rule for the influence of valency upon coagulation. It is worthy of notice that the same rule seems to hold for the NH_4 salts. If we compare again the minimal concentrations which allow about 70 per cent f the eggs to recover, we find that the values are M/32 for NH_4Cl, /128 for $(NH_4)_2SO_4$ and M/512 for $(NH_4)_3$ citrate which is gain Cl : SO_4 : citrate = 1 : 4 : 16. The values are practically dentical with those found for the Na salts, thus showing that re are not dealing with an action of the salts upon the heart ince the NH_4 salts are unable to maintain or resuscitate the eart beat.

Table VII gives the influence of the cations upon the recovery.

TABLE VII.

	Percentage of embryos poisoned by KCl recovered after 24 hrs.													
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	M/4,096	H_2O
iCl		30	45	80	60	80	60	30	5	0	10			5
aCl	90	100	100	100	100	90	40	25	15	10				0
bCl		0	0	0	5	5	0	1	0	2	0			0
sCl		5	5	0	0	5	5	0	0	0	0			
gCl_2			100	85	95	75	55	25	25	25	15	10		5
aCl_2				100	100	75	45	20	20					
rCl_2				30	15	30	15	20	0	5	25	0	0	10
aCl_2			5	0	5	10	15	25	10	20	35	10	20	0
H_4Cl		38	72	89	83	77	41	16	15	15				11
$(C_2H_5)_4Cl$					100	78	60	23	20	14	5	14	12	10

In this case a peculiar influence of the periodic law is noticeable. i and Na as well as the corresponding members of the next roup Mg and Ca favor the recovery, Rb and Cs as well as the orresponding members Sr and Ba inhibit it. NH_4Cl and $(C_2H_5)_4Cl$ act like NaCl. It is strange that Mg and Ca should crease the permeability in the recovery experiments and have he opposite effect in the case of the diffusion of KCl into the egg. he fact that salts which cannot reestablish the heart beat or aintain it like $N(C_2H_5)_4Cl$ should have almost as powerful an ction upon the recovery of the hearts as Na shows that we are ealing with an effect of the salts upon the membrane and not pon the protoplasm of the embryo.

CONCLUSION.

These experiments confirm the conclusion that for the diffusion of the potassium salts through the membrane, aside from the osmotic pressure of the solution, a second factor is required, the general salt effect upon the membrane. While this was proved, in the first part of this series, for the diffusion of potassium salts *into* the egg, it is proved in this part for the diffusion in the opposite direction. It is shown that eggs previously poisoned with KCl cannot recover when put into the solution of any non-electrolyte. A few eggs may recover in such a solution at the beginning, due to the fact that at the beginning the external surface of the egg may have enough KCl for the salt effect upon the membrane. As soon as this salt has diffused away from the surface, no further recovery is possible, since the external surface of the membrane when sufficiently freed from salts is as efficient a barrier for the diffusion of KCl out of the egg as in the opposite direction.

The relative efficiency of different salts for the salt effect upon the membrane is a function both of anion and cation. The efficiency increases with the valency of the anion approximately according to Hardy's rule, Cl : SO_4 : citrate = 1 : 4 : 16, as we had stated already in previous notes; but the efficiency also depends upon the nature of the anion in the following way:

$$Cl, NO_3 < Br < Acetate < PO_4, CO_3 < SO_4 < Citrate.$$

In regard to the cation we found that Rb and Cs completely inhibit the diffusion of KCl cut of the egg in M/2 solution. The salts of Sr and Ba have a similar effect. The salts of Na, Li, Mg, Ca, NH_4, and $N(C_2H_5)_4$ favor the diffusion. Since the salts of Li, NH_4, and $N(C_2H_5)_4$ are extremely toxic for the embryo the beneficial influence of these salts upon the recovery from potassium poisoning must be ascribed to an action upon the membrane and not to an action upon the heart of the embryo. Since the balanced solutions are almost as efficient as non-balanced solutions, the influence cannot consist in a destructive action upon the membrane, but must consist in the creation of special conditions required for the diffusion of electrolytes.

THE MECHANISM OF THE DIFFUSION OF ELECTROLYTES THROUGH THE MEMBRANES OF LIVING CELLS.

III. THE ANALOGY OF THE MECHANISM OF THE DIFFUSION FOR ACIDS AND POTASSIUM SALTS.

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I.

We shall show in this paper that the ideas concerning the rôle of the general salt effect developed in the two previous notes[1] hold also for the diffusion of acids into the egg of *Fundulus*. The effect of acids upon the heart of *Fundulus* is not to the same degree reversible as the effect of potassium salts, since when acid diffuses into the egg it first kills and causes coagulation of the body of the embryo from the tail upward and only later causes the heart to stop beating by diffusion through the pericardium. It would be difficult if not impossible to demonstrate the influence of salts upon the recovery of the heart from acid poisoning. We must, therefore, make use of the diffusion of acids *into* the egg for the demonstration of the salt effect in the case of acids. We shall show that eggs washed in H_2O are more resistant to acid than eggs containing some salt. For this demonstration the eggs were first put for 4 days into salt solutions of a different concentration and then exposed to the acid solution. Eggs similarly treated were also exposed to M/8 KCl solutions with this difference only, that it was found sufficient to expose the eggs for 1 day only, previous to their transfer to M/8 KCl. Since the experiments with KCl were more numerous than those with acid, we shall describe them first.

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[1] Loeb, J., *J. Biol. Chem.*, 1916, xxvii, 339, 353.

The method employed in the case of the potassium experiments was as follows: The eggs were put for 24 hours into solutions of certain substances varying in concentration. A chemical equilibrium is established between the constituents of the solution and the external surface or layer of the membrane. The inner layer of the membrane is not at all or only little affected. These eggs are then put into an M/8 KCl solution. The hearts will stop beating and the rate at which this happens will give us the rate at which the KCl diffuses. The smaller the percentage of the hearts beating at a certain time, the greater the rate at which the KCl diffuses into the eggs.

For the understanding of these results we must remember that when the egg is transferred from a salt solution into a pure M/8 KCl solution the salts other than KCl will diffuse from the external surface or layer of the membrane into the KCl solution and the greater part of this diffusion will take place in the first hours. We have shown in the first paper of this series that when two lots of eggs are put for 24 hours, one into sea water and the other into H_2O, and then both are transferred to M/8 KCl the main difference in the rate of diffusion of KCl through the membranes of the two lots of eggs will be found in the first 6 hours, since during this period the eggs from the sea water will still have enough salt at the surface of their membranes to supply the salt effect required for the diffusion of KCl through the membrane. Hence more of the eggs taken from sea water will be poisoned during the first 6 hours than of the eggs taken from H_2O; while *after* the first 6 hours the further rate of poisoning will be more nearly the same for the two groups of eggs.

Eggs were put for 24 hours into various concentrations of sea water and then exposed to an M/8 KCl solution. Table I gives the percentage of embryos whose hearts were beating after different intervals in the KCl solution.

The result is quite striking. The eggs taken from normal sea water had in half of the cases enough salt at the surface of their membranes to allow so much KCl to diffuse into the eggs inside of 3 hours that the hearts stopped beating. Of the eggs taken from M/4 sea water only 15 per cent had enough salt at the surface of their membranes to allow the same result. The eggs taken from M/8 or still more diluted sea water behaved like the

TABLE I.

After	Percentage of embryos with beating hearts in M/8 KCl taken from sea water or H_2O.								
	Sea water.								H_2O
	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	
hrs.									
3	55	85	100	100	100	100	100	100	100
5½	50	85	100	100	100	100	100	100	100
8	45	85	100	100	100	100	100	100	100
25	50	85	95	95	100	95	100	100	100

eggs that had been in distilled water, inasmuch as they had not enough salt left at their external surface to permit the diffusion of enough KCl into the egg to cause cessation of heart beat in a single egg (Table I).

It seemed advisable to see what would happen if the experiments were made with eggs kept in balanced solutions of higher concentrations than that of sea water (M/2). Eggs were put for 2 days into mixtures of NaCl + $CaCl_2$ in the proportion of 100 molecules of NaCl to 1.75 molecules of $CaCl_2$ (as in sea water) varying in concentration from 3 M to M/128. From these solutions the eggs were transferred into an M/8 solution of KCl and the percentage of eggs with beating hearts was ascertained after certain intervals (Table II).

This experiment shows that eggs which had been in M/32 NaCl + $CaCl_2$ or in lower concentrations contained so little salt at their surface that the KCl could not diffuse any more rapidly

TABLE II.

After	Percentage of eggs with beating hearts in M/8 KCl taken from NaCl + $CaCl_2$.											
	3 M	2½M	2 M	1½M	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128
hrs.												
3	90	65	0	0	0	0	0	30	80	100	100	100
5½	40	15	0	0	0	0	0	25	75	100	100	100
8	5	5						25	75	100	100	100
25	0	0	0	0	0	0	0	30	75	90	95	95
75	0	0	0	0	0	0	0	15	55	85	80	95

into the egg than if the eggs had been kept in H_2O. The eggs taken from M/16 NaCl + $CaCl_2$ had at the beginning enough salt at their surface so that 20 per cent of the eggs were poisoned in the first 3 hours. In the meantime the diffusion of the NaCl + $CaCl_2$ from the surface of the membranes took place so rapidly that afterwards no more eggs were poisoned in the next 25 hours and only a few more in the following 2 days. The eggs taken from the M/8 solution of NaCl + $CaCl_2$ had enough salt at their surfaces to allow the diffusion of a poisonous dose of KCl into 70 per cent of the eggs in the first 3 hours, and in the meantime so much of the $CaCl_2$ + NaCl left the surface of the membrane that the eggs from then on behaved like washed eggs, so that in the next 25 hours the number of eggs poisoned did not increase, and increased only slightly in the next 2 days. All the eggs that had been in the next higher solutions, from M/4 to 2 M, were completely poisoned by M/8 KCl in less than 3 hours, which means that they had enough salt at the surfaces of their membranes to allow the diffusion of a poisonous dose of potassium through the membrane in less than 3 hours. The eggs, however, that had been in 2½ and 3 M NaCl + $CaCl_2$ show another picture; they had an excessive amount of salt at the external surface of their membranes which made them for a number of hours immune against the M/8 KCl solution (antagonistic salt action). This immunity did not last long, however, since the Na and Ca diffused from the surface of the membrane into the M/8 KCl solution and then after about 5 hours a stage was reached when the eggs had only that moderate amount of salt at the external surface of their membrane which accelerates the diffusion of a toxic dose of KCl into the egg. This happens more quickly in the case of the eggs that had been kept for 2 days in a 5/2 M than in a 3 M solution. The same fact, that a moderate amount of salt at the surface of the egg is required to permit the diffusion of KCl into the egg while a higher concentration prevents the diffusion, is shown in the following experiment. Eggs had been put for 2 days into a mixture of NaBr + $CaCl_2$ (in the same proportion as in sea water) and were then transferred into M/8 KCl. Table III gives the percentage of eggs whose hearts stopped beating after several hours.

Again we see that a previous treatment with a moderate concentration (M/8 to M) of a balanced solution accelerates the rate of diffusion of KCl into the egg while previous treatment with a more concentrated solution retards it. But this retardation lasts only through the first hours, as long as the NaBr has not diffused away from the surface of the membrane. It is noteworthy that the inhibiting or antagonistic concentration is lower for NaBr + $CaCl_2$ than for NaCl + $CaCl_2$, suggesting possibly a firmer attachment to the membrane in the case of NaBr than in the case of NaCl. It is also obvious that the general salt effect of sea water is less than that of NaCl + $CaCl_2$ or of NaBr + $CaCl_2$ solutions of equal concentration. This difference must be due to the presence of some of the other constituents in sea water than NaCl and $CaCl_2$.

TABLE III.

After	Percentage of eggs with beating hearts in M/8 KCl after having been kept for 48 hrs. in NaBr + $CaCl_2$ or H_2O.									
	NaBr+$CaCl_2$									H_2O
	2 M	1½ M	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	
hrs.											
3	81	15	0	0	0	15	70	100	100	100	100
5½	60	10	0	0	0	15	65	95	100	100	
8	20	5	0	0	0	10	65	95	100	100	
25	0	0	0	0	0	10	60	90	100	100	
75	0	0	0	0	0	5	45	70	85	80	

Considering the theoretical importance of these experiments, one more observation of the same type may be recorded, in which the eggs had been put for 17 hours into various concentrations of a Ringer solution (NaCl + KCl + $CaCl_2$) and for controls in M/2 sea water and H_2O. They were then transferred into a M/2 KCl solution (instead of M/8, as in the experiments thus far mentioned). Table IV gives the results.

As in the preceding experiments we notice that the previous treatment with normal (M/2) sea water accelerates the diffusion of KCl more than a previous treatment with a M/2 Ringer solution. Again we notice that a moderate concentration of the Ringer solution, from M/16 to M/2, accelerates the diffusion more than a higher concentration, like 5 M/2. The difference which is very striking during the first hours becomes less in time for two reasons; first, that the salt at the surface of the membrane diffuses into the solution, and second, that in as high a concentration as M/2 the KCl solution itself rapidly supplies the salt effect necessary for the diffusion of its own molecules or ions through the membrane.

The demonstration of the general salt effect in the case of the balanced solutions had the advantage of proving that this effect does not consist in an injurious action of the salt upon the membrane. Since M/2 sea water is the ideal balanced solution and the natural milieu in which these eggs develop, it cannot be said that eggs taken from normal sea water or from M/2 NaCl + $CaCl_2$ succumb more rapidly to KCl because the natural sea water hurts the membrane more than dilute sea water or H_2O. As a fact the contrary is more nearly correct.

TABLE IV.

After	Percentage of eggs with beating hearts in M/2 KCl after having been kept for 17 hrs. in Ringer solution, sea water, or H_2O.												
	Ringer solution.											M/2 Sea water.	H_2O
	5M/2	4M/2	3M/2	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128		
hrs.													
2	90	80	90	65	20	30	65	85	100	95	85	5	100
4	60	30	30	25	15	15	55	70	95	95	85	0	100
6	35	10	5	15	10	10	45	65	95	85	75	0	85
8	15	0	0	15	5	10	30	45	70	70	55	0	75
12	5	0	0	5	0	5	5	30	50	30	35	0	50

Eggs were put for 24 hours into non-balanced solutions of various sodium salts of concentrations varying from M/1 to M/1,024 or less. They were then put into M/8 solutions of KCl. Only eggs with beating hearts were put into the KCl solution. Table V gives the percentage of beating hearts after 6 hours in the different solutions.

The difference in the efficiency of the anions in the production of the general salt effect is quite obvious. When we select for comparison the highest concentration of each salt which produced

TABLE V.

	Percentage of eggs with beating hearts in M/8 KCl after 6 hrs. when kept previously for 24 hrs. in												
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	M/2,048	H_2O
NaCl........	0	0	0	16	100	100	100	100	100	100	100		100
NaBr........	0	0	5	0	84	100	100	100	100	100	100	100	100
Na acetate...		0	0	30	80	100	100	100	100	100	100	100	100
Na_2SO_4.......		0	0	0	74	90	100	100	100	100	100		100
Na_3 citrate...								82	93	96	100	100	100
Na_2HPO_4....								42	67	100	100		100

no more salt effect, *i.e.*, that concentration which rendered 90 per cent of all the eggs immune against the M/8 KCl solution in the next 6 hours, we notice the following results:

NaCl..	M/ 16
NaBr..	M/ 32
Na acetate..	M/ 32
Na_2SO_4..	M/ 64
Na_3 citrate..	M/256
Na_2HPO_4..	M/512

Above these concentrations these salts produced the salt effect. The ratio of the limiting concentration for the salt effect of Na salts is therefore Cl : SO_4 : citrate or phosphate approximately as 1 : 4 : 16, the same as that found in the recovery experiments (second paper of this series).

It might be argued that the hearts stopped beating after the treatment with M/2 NaCl or NaBr or M/4 Na_2SO_4 not on account of the injurious effect of the M/8 KCl but on account of the toxic effects of the NaCl or Na_2SO_4 solutions. This argument would be wrong since only such eggs were transferred to the M/8 KCl solution whose hearts were beating after having been 24 hours in M/1, M/2 NaCl, or M/2, M/4, or M/8 Na_2SO_4, or any of the other solutions, and, moreover, we had found in the second paper that the relative efficiency of these salts to induce the recovery from potassium is exactly the same as that for poisoning in these experiments. Moreover we convinced ourselves that eggs which had been poisoned in M/8 KCl after a treatment in M/4 Na_2SO_4 recovered when put back into the latter solution although M/4 Na_2SO_4 rapidly kills the embryo outside the egg.

The effect of the cation is equally marked in Table VI, showing the efficiency of different chlorides. The eggs were put for 24 hours into the solutions of various concentrations of these salts; the concentrations were below that limit in which they kill or injure the egg in 24 hours. After 24 hours the eggs were put into an M/8 KCl solution. The percentage of eggs with heart beats is given in Table VI.

TABLE VI.

	Percentage of eggs with beating hearts in M/8 KCl after 6 hrs. which had previously been treated for 24 hrs. with										
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024
LiCl............		70	85	47	89	90	100	100	100	100	100
NaCl............	0	0	0	15	100	100	100	100	100	100	100
RbCl............		33	88	96	100	100	100	100	100	100	100
CsCl............			50	90	100	100	100	100	100	100	100
NH_4Cl............			85	85	90	95	100	100	92	100	100
$MgCl_2$............			85	100	100	100	100	100	100	100	100
$CaCl_2$............				89	100	100	100	100	100	100	100
$SrCl_2$............				96	100	100	100	100	100	100	100

This table reveals the unexpected fact that Na has the most striking salt effect of all the cations mentioned. Since NaCl is also the least toxic of all the salts and since it injures the membrane less than any of the other salts mentioned in the table, it is obvious that the acceleration of the diffusion of KCl through the membrane cannot be ascribed to an injurious effect of the salt on the membrane.

A previous treatment of the eggs with the solutions of non-electrolytes does not lead to an acceleration of the diffusion of the KCl through the membrane. Eggs were put for 24 hours into solutions of various non-electrolytes in different concentrations. Nothing comparable to the salt effect was produced; the eggs behaved as if they had been washed in H_2O.

It is obvious that the membrane of eggs which had been for 24 hours in a solution of a non-electrolyte, *e.g.*, glucose, cane sugar, glycerol, and methyl alcohol had become impermeable to M/8 KCl to the extent that in 6 hours not enough KCl could diffuse into the egg to cause cessation of the heart beat in more than 20 per cent of the eggs. The non-electrolytes act, therefore, essen-

TABLE VII.

	Percentage of eggs with beating hearts after treatment for 6 hrs. in M/8 KCl. Eggs previously kept for 24 hrs. in											
	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024	H_2O
lucose..........		96	95	97	95	90	96	100	100	100		100
ane sugar......	81	96	100	100	100	100	100	100	100	100	100	100
lycerol..........	81	90	90	90	95	96	100	100	100	100		100
ethyl alcohol...	84	100	100	100	100	100	100	100	100	100	100	100

tially like distilled water unless they are applied in excessive concentration. In that case we may expect an "injurious" action on the membrane, different both from the antagonistic action and the general salt effect upon the membrane. This phenomenon will be discussed in the fourth paper of this series.

II.

We shall now try to show that for the diffusion of acids through the membrane of the *Fundulus* egg, beside the osmotic pressure, a salt action upon the membrane is also required. The difference between the two cases is that acids can produce the salt effect in a concentration at least several hundred times smaller than the concentration required for the same purpose in the case of KCl. Thus M/1,000 acetic acid kills the egg rapidly by coagulating the embryo. In order to demonstrate the salt effect it was necessary to show that when the membranes of the egg were freed from salt by previous washing in H_2O, it took longer to kill the embryos than when eggs taken directly from sea water were exposed to the acid. The writer tried in vain last year to furnish this proof. He found this year the reason for the failure; he had washed the eggs for 24 hours in H_2O (as in the potassium experiments). This washing probably left a trace of salt on the external surface or the external layer of the egg membrane which was negligible for the diffusion of potassium salts but which was sufficient to prevent the demonstration of the salt effect in the case of acids. When eggs were washed for 4 days in H_2O it was possible to demonstrate the salt effect for acids too. The method of the experiment was as follows: Eggs were put for 4 days into H_2O and into different concentrations of NaCl + KCl

+ $CaCl_2$ (in the proportion of 100 molecules NaCl to 2.2 molecules KCl to 1.75 molecules of $CaCl_2$ as in sea water). They were then put into 50 cc. of M/1,000 acetic acid. In acid solutions the embryo is killed by coagulation and becomes white. The coagulation shows itself first in the tail of the embryo and then creeps upward. The heart is the last organ to be affected by the acid, probably because it is protected by the pericardium. Thus the tail of the embryo is already opaque when the heart ceases to beat. The cessation of heart beat in this case means death of the embryo. Table VIII gives the percentage of embryos whose hearts were still beating.

TABLE VIII.

After	Percentage of embryos surviving in M/1,000 acetic acid after having been kept for 4 days in the following solutions.											
	NaCl + KCl + $CaCl_2$										H_2O	M/2 Sea water.
	3M/2	M/1	M/2	M/4	M/8	M/32	M/128	M/512	M/1,024	M/2,048		
hrs.												
5	100	100	90	90	75	85	100	90	95	95	100	85
7	95	95	50	65	35	55	70	75	85	85	70	60
8	85	60	30	45	35	35	45	70	65	85	65	35
9	80	50	25	30	25	30	15	70	50	70	50	15
11	45	35	10	15	5	30	5	55	35	35	35	5
23	20	10	0	5	0	5	0	5	0	0	5	5

We must remember that the salt in combination with the surface from the previous treatment diffused during the first hours into the surrounding acid solution which was free from salts. We must therefore look for differences in the effects of the previous treatment of the eggs during the first hours of the experiment. After 5 hours all the embryos which had been in H_2O and 3 M/2 and M/1 Ringer were still intact in the M/1,000 acetic acid solution, while 25 per cent of those that had been in M/8 Ringer and 15 per cent of those that had been in sea water were already dead. After 7 hours two-thirds of those that had been in M/8 Ringer were already dead and not yet one-third of those which had been in distilled water had been killed. After 8 hours the accelerating salt effect upon the diffusion of acid through the membrane was still more striking; those that had been in

M/2 to M/32 Ringer or M/2 sea water died twice as fast as those that had been in H_2O. After 23 hours the effect of the previous treatment had disappeared since the salts had diffused from the surface of the membrane.

Hence we see that in the case of acids as in the case of KCl, a small dose of salt on the external surface of the membrane accelerates the diffusion, a larger dose retards the diffusion. In eggs washed in H_2O the acid itself supplies the salt effect upon the membrane, much less acid than neutral salt being required for this purpose.

On account of the theoretical importance of the subject, a second experiment of the same type may be reported. The eggs were put again for 4 days into the solution before being transferred to the M/1,000 acetic acid solution. Table IX gives the result.

TABLE IX.

After	Percentage of eggs with beating hearts in M/1,000 acetic acid after having been kept for 4 days in the following solutions.														
	NaCl + KCl + $CaCl_2$													H_2O	Sea water.
	5M/2	2M	3M/2	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512		
hrs.															
3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
6	100	100	100	87	65	40	61	80	70	85	85	90	88	94	55
7	93	94	100	25	20	25	20	39	20	35	70	55	66	82	30
8	76	88	95	15	5	5	9	18	5	35	40	45	48	56	20
9	76	70	80	10	0	5	5	18	0	25	30	40	30	24	10
11	48	48	33	0	0	0	0	5	0	15	10	25	10	19	5
23½	15	6	14	0	0	0	0	0	0	5	0	5	5	0	0

A glance at the table will show that the eggs previously treated with a moderate concentration of salts, M/32 to M/1, or with sea water, are killed by the acid more quickly than the eggs previously treated with distilled water, while those treated with a higher concentration of the salt die more slowly (antagonistic salt action). These experiments show incidentally that the antagonistic salt action (or the general salt effect) occurs between the membrane (modified by the antagonistic salt) and the acid, and that it is immaterial whether the antagonistic salt is applied simultaneously with the injurious salt or previously, except that the antagonistic salt action cannot last in the latter case on account of the diffusion of the salt from the membrane into the solution.

When the eggs were washed for 1 or 2 days only, the antagonistic salt action due to the previous treatment with the stronger solutions was more marked than the accelerating action of the more moderate salt concentrations as the following example shows (Table X).

TABLE X.

After	Percentage of eggs with beating hearts in M/1,000 acetic acid after having been kept for 2 days in the following solutions.															
	$NaCl + KCl + CaCl_2$														H_2O	
	5M/2	2 M	3M/2	M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1,024		
hrs.																
5	100	90	95	85	85	15	25	25	25	35	35	25	55	35	45	50
9	85	45	50	55	30	0	5	15	5	0	10	5	10	10	10	10
20	25	20	15	0	0	0	0	0	0	0	0	0	0	5	0	0

In this experiment the antagonistic effect of the salts (which were left on the surface of the membrane) on the diffusion of acid is very striking, especially in the eggs previously washed in 5 M/2, 2 M, and 3 M/2 $NaCl + KCl + CaCl_2$. A slight accelerating effect of the salt is only noticeable in the eggs previously washed in M/4 $NaCl + KCl + CaCl_2$. The washing was too short to remove enough salt from the surface of the egg in the H_2O so that the trace of salt left on the surface of the membranes of the eggs was still sufficient to supply the general salt effect. The diffusion of acids requires a much lower concentration of a second salt at the external surface of the membrane than the diffusion of KCl, possibly because acid forms a much more stable salt with certain proteins than neutral salts (Hardy); as a consequence, a trace of acid can by combining with the membrane (or its proteins) produce the same salt effect as a considerable concentration of a neutral salt.

We have already mentioned in a previous note[2] that the antagonistic action of salts to acid in the case of the *Fundul* embryos increases not only with the valency of the anion but with that of the cation as well, which is also the case when KCl is antagonized by other salts. We shall return to this fact in another connection, though we may add here that this should put an end to such generalizations as that the antagonistic salt action is due to the oppositely charged ions.

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[2] Loeb, *J. Biol. Chem.*, 1915, xxiii, 139.

CONCLUSION.

This paper shows that under certain experimental conditions the existence of the general salt effect can be demonstrated also for the diffusion of acid through the membrane of the egg of *Fundulus*. The concentration of neutral salt required for the salt effect is considerably smaller in the case of the diffusion of acid than in the case of the diffusion of potassium salt. Very weak acid solutions themselves can supply the general salt effect, owing probably to the fact that acids form stable salts with certain proteins of the membrane (while neutral salts form only unstable salts).

When the concentration of neutral salt added to the acid is a little higher than that required for the salt effect the opposite phenomenon is produced; namely, the diffusion of acid is retarded or inhibited (antagonistic salt action).

It is shown that under the conditions of these experiments similar rules hold for the diffusion of potassium salts through the membranes of living eggs of *Fundulus*.

THE FATE OF ALKALI BLUE IN THE ORGANISM.

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The excretion of lipoid-insoluble dyes after parenteral administration into frogs has been investigated by Höber and Kempner. They concluded that the more characteristically colloidal the state of the dye the more difficult is its passage through the kidneys. Alkali blue (*Alkali-blau*), which is a highly colloidal compound, was not eliminated through the kidneys.

The fate of various types of dyes in the organism is of interest in the study of the permeability and secretory power of blood vessels and glands. At the suggestion of Professor Lafayette B. Mendel, I have investigated the behavior of alkali blue in the organism. All necessary operations on animals were performed by him.

Methods.

Alkali blue—sodium triphenylrosaniline monosulfonate—is soluble in cold water, much more so in alcohol and in warm water, but insoluble in ether and in oil. It is precipitated by calcium or lead salts. The color is decreased by dilute ammonia, and it can be restored with acids. The dye (Kahlbaum's) used in the following experiments was dissolved in distilled water in the proportion of 1 or 2.5 per cent.

The method for the detection of the dye in tissues and in body liquids was investigated in preliminary experiments. The intensity of blue color decreases when the dye is passed through the animal body or kept a little while with body liquids. On addition of a small amount of acetic acid, the color reappears, but not always to the original intensity. Boiling or addition of hydrogen peroxide seemed to cause no improvement. Though the exact nature of the change is yet unknown, a similar alteration of color has been noted with many other dyes. Underhill and Closson demonstrated that methylene blue, administered to an animal, reappears in the urine and feces in four forms—methylene blue itself, methylene azure, *i.e.*, an oxidation product of methylene blue, and leuko compounds corresponding to methylene blue and methylene azure. As these leuko compounds are reduction products of either methylene blue or methylene azure, methylene blue must undergo both oxidation and reduction in the animal body. Indigo carmine, rosaniline, and phenoltetrachlorphthalein, introduced into the organism, reappear in both free and leuko states. Bulk demonstrated that alkali blue becomes colorless by reducing agents, such as ammonium sulfide, the color being called forth again by oxidation.

For the detection of alkali blue in lymph, the material was slightly acidified with acetic acid, whereupon the blue color developed or deepened in a few minutes. In order to demonstrate the presence of the dye in blood, four volumes of 95 per cent alcohol were mixed with one volume of blood sample. The mixture was stirred vigorously, allowed to stand for 2 hours, and filtered. The filtrate was made faintly acid with acetic acid, whereupon the maximum color developed. For the detection of alkali blue in organs, tissues, or in dry feces, the material was ground in a mortar with 95 per cent alcohol in the proportion of 5 cc. alcohol for each gram thereof. The mixture was allowed to stand for 2 hours in a stoppered bottle. The filtrate was then treated as in the case of blood. To detect the dye in the urine, acetic acid was added without any other manipulation. The presence of the dye in the bile was easily demonstrated by acidifying. The result was especially clear when the bile was diluted with about 200 volumes of water before acid addition. In order to make an approximate estimation of the dye in the feces, the whole material was diluted to 1 or 2 liters. 50 cc. of it were mixed with neutral lead acetate, the latter being added until precipitation ceased. The sediment containing the whole amount of the dye was placed in a 100 or 250 cc. measuring flask, mixed with a few cc. of acetic acid, and made up to the mark with 95 per cent alcohol. The filtrate was then compared in a Duboscq colorimeter with the standard, prepared from the original dye solution by acidifying and diluting with alcohol.

PROTOCOLS.

Experiments with Dogs.

Experiment I.—Dog 1, male, 8.8 kg., was anesthetized with ther after injection of 90 mg. of morphine and 9 mg. of atropine. cannula was introduced into the thoracic duct; and 8 cc. of 2.5 er cent solution of alkali blue were injected into the femoral vein.

TABLE I.

Lymph.

dministration of dye and diuretic.	Lymph flow in 10 min. intervals.		Blue color.	
	Sample No.	Amount.	Before acidification.	After acidification.
		cc.		
	Before injection.	4.2	—	—
cc. of alkali blue into femoral vein.				
	1	4.2	—	—
	2	2.9	—	+
	3	2.9	—	++
	4–7	2.8–4.4	—	++
cc. of alkali blue into femoral vein.				
	8–10	3.3–4.6	—	++
	11–12	4.0–4.6	+	+++
50 cc. of 0.9 per cent NaCl into femoral vein.				
	13–20	9.0–10.4	+	+++
	21–24	5.0– 7.4	+	+++

*Blood.**

Time after first dye injection.	Blue color.	
	Before acidification.	After acidification.
min.	±	+++
0 "	—	++
hr.	—	++
" 30 min.**	±	+++
hrs.	—	++
"	—	+

* Samples were taken from the femoral artery.

** 20 min. after the second injection of the dye.

Urine.—Samples, taken by aspiration of the bladder 1, 2, 3, and 4 hours after the first injection of the dye, did not contain the dye in a free form, but showed a very pale blue color when acidified with acetic acid. The urine flow was very slow.

Autopsy.—4 hours after the first injection of the dye, the dog was killed by bleeding. A strong blue color of the liver was the most noticeable feature observed. The other organs and tissues showed no such abnormal color. The alcohol extract of the liver appeared intensely blue. Acid addition made its color much brighter. The bile in the gall bladder contained the blue dye. The pyloric portion of the stomach mucosa was covered with intensely blue mucus, the other parts of the stomach showing the normal color. The blue color extended to the greenish blue mucus in the duodenum. The blue color of the latter was markedly increased by acidifying, but the color of the former showed no such intensification. The mucus in the upper part of the jejunum was brownish yellow and showed a slight blue color by acid addition. Further downwards such development of blue color by acid addition could not be demonstrated. Even where the stained mucus was contained the mucous membrane itself showed no abnormal color. The kidneys showed no evidence of blue color. In the alcohol extracts a blue color could not be observed, even after acidification.

Experiment II.—Dog 2, female, 7.7 kg. First a cannula was introduced in the thoracic duct, and before the conclusion of the experiment a bile fistula was established in the common bile duct. As narcotics urethane (10 gm.) and ether were used.

TABLE II.

Lymph.

Administration of dye and diuretic.	Lymph flow in 10 min. intervals.		Blue color.	
	Sample No.	Amount.	Before acidification.	After acidification.
		cc.		
	Before injection.	1.4	–	–
8 cc. of alkali blue into femoral vein.				
	1	1.4	–	±
	2	3.1	+	++
	3–5	2.9–5.4	+	+++
0.15 gm. of caffeine citrate and 100 cc. of 0.9 per cent NaCl into femoral vein.				
	6–11	2.0–4.8	+	+++
	12–18	0.7–1.6	+	+++

TABLE II.—*Concluded.*

*Blood.**

Time after the dye injection.	Blue color.	
	Before acidification.	After acidification.
5 min.	±	+++
50 "	−	++
1 hr. 50 min.	−	++
3 hrs.	−	+

* Samples were taken from the femoral artery.

Urine.—5, 85; and 180 minutes, respectively, after the dye injection, urine samples were obtained by catheterization. In none of them was the dye found. Though caffeine and saline solution were given, the urine secretion was very slow.

Bile.—3¼ hours after the dye injection, a cannula was inserted into the common bile duct. The bile flow in successive 10 minute periods was 0.9, 1.1, and 0.5 cc. The color of these samples was always dark blue. When kept a few hours, their color became yellowish green, but an addition of one drop of acetic acid called forth again the dark blue color.

Autopsy.—4 hours after the dye injection, the dog was killed by bleeding. The observations were essentially similar to those described for Dog 1, the exceptions being that the dye was not found in the stomach and duodenum, but it was abundant in the greater part of the jejunum and ileum.

Experiment III.—Dog 3, female, 9.5 kg. Cannulas were introduced into both the thoracic duct and the common bile duct, as in Dog 2. To induce a marked urine flow, a large amount of the saline solution was injected.

Urine.—Of the samples taken from time to time by catheterization, only one, removed 1½ hours after the dye injection, contained a trace of the dye, the others always yielding negative results. Though a large amount of the saline solution was injected, the urine flow was not augmented. Protein and casts were found in some samples.

Bile.—2 hours after the dye injection, a fistula was established in the common bile duct. The bile samples, collected during

TABLE III.

Lymph.

Administration of dye and diuretic.	Lymph flow in 10 min. intervals.		Blue color.	
	Sample No.	Amount.	Before acidification.	After acidification.
		cc.		
	Before injection.	4.8	−	−
8 cc. of alkali blue into femoral vein.				
	1* Part 1	2.0	−	−
	" 2	1.2	−	±
	" 3	0.8	−	+
	" 4	0.9	+	++
	2- 3	4.0-2.6	+	++
400 cc. of 0.9 per cent NaCl into femoral vein.				
	4- 5	3.3- 5.5	+	+++
	6-12	8.5-13.0	+	+++
	13-18	4.4- 8.0	+	+++

*Blood.***

Time after the dye injection.	Blue color.	
	Before acidification.	After acidification.
50 min.	−	++
1 hr. 30 min.	−	+
2 hrs.	−	+
2 " 30 min.	−	+
3 "	−	±

* Subdivisions were collected during each 2½ min.

** Samples were taken from the femoral artery.

successive 10 minute periods, measured 1.2, 1.3, 1.5, 1.0, 0.7 and 0.8 cc., respectively. The color was greenish blue and b haved as described for Dog 2.

Autopsy.—3 hours after the dye injection, the animal was killed by ble ing. The observations were essentially similar to those described for I 1. In the alimentary tract the dye was found in the pyloric portio the stomach, the duodenum, and the jejunum.

Experiment IV.—Dog 4, male, 11.2 kg., was used to see whe alkali blue can enter the lymph otherwise than through the ymphatics. As a narcotic ether was used. A cannula was placed n a cervical lymph duct. The lymph flow, facilitated by gentle massage, was very slow. 8 cc. of 2.5 per cent solution of alkali blue were injected into the femoral vein. The cervical ymph, taken within 10 minutes after the dye injection, showed no blue color, even by acid addition. The samples, taken later, changed to blue after acidification; without this manipulation the color was pale yellow. 30 minutes after the dye injection a lymph sample was taken from the thoracic duct. It had a pale green color, and after acid addition it developed an intense blue color, which was much deeper than that developed by acidifying the cervical lymph sample taken at the same time. This shows that the thoracic duct lymph, derived largely from the iver lymphatics, contained the dye in more concentrated form than the cervical lymph. 45 minutes after the dye injection the dog died suddenly. The bile, taken from the gall bladder, was yellow-brown and did not show any change of color by acid addition. Though the color change of the liver was not intense, an alcohol extract contained the dye in free form. The blood contained the dye only in the leuko state. The protein-free urine contained no dye.

Experiment V.—Dog 5, male, 9.5 kg., was used for comparison, phenoltetrachlorphthalein and indigo carmine being the dyes injected. After a dose of morphine (95 mg.) and atropine (9.5 mg.), the dog was anesthetized with ether.

Urine.—The sample, taken 1½ hours after the phenoltetrachlorphthalein injection, became faintly red by NaOH addition. The samples, taken 30 and 70 minutes after the indigo carmine injection, were so dark blue with the dye that they could not be tested for the red dye.

Autopsy.—The mucous membrane of the mouth was markedly blue. All viscera were stained a blue color. The liver was also blue, but not so ntense as seen in the experiments with alkali blue. The bile in the gall bladder was markedly blue. The stomach mucosa showed nothing abnormal. The duodenum contained blue mucus. Its color became a ittle stronger by acid addition. Moreover, with NaOH addition a strong red color appeared. The first part of the jejunum contained brownish yellow mucus. No color change was seen by acidification, but a slight red color developed after alkali addition. Further down no color change was produced either by acid or alkali addition.

TABLE IV.

Lymph.

Administration of dye.	Lymph flow in 10 min. intervals.		Red color.		Blue color.	
	Sample No.	Amount.	Before alkali addition	After alkali addition.	Before acid addition.	After acid addition
		cc.				
	Before injection.	1.8	–	–		
8 cc. of 2.5 per cent phenoltetrachlorphthalein into femoral vein.						
	1	3.5	–	±		
	2	1.4	–	+		
	3–14	1.1–5.4	–	++		
20 cc. of 1 per cent indigo carmine into femoral vein.						
	15	8.0			+*	++—
	16	8.0			++	++–+
	17–21	4.8–9.6			++	++–+

Blood.†

Time after phthalein injection.	Red color.		Blue color.	
	Before alkali addition.	After alkali addition.	Before acid addition.	After acid addition.
10 min.	–	++		
50 "	–	+		
2 hrs. 10 min.	–	±		
2 " 40 " **	–	±	–	+
3 "	–	–	–	–

* The first appearance of the dye in a free state was 6 min. after t dye injection.

† Samples were taken from the femoral artery.

** 20 min. after indigo carmine injection.

Experiment VI.—Dog 6, male, 5.0 kg., was used to see whe alkali blue is eliminated into the alimentary tract othe than through the bile duct.

The day before the operation no food was given. As narcotics rethane (3.8 gm.) and ether were used. A temporary bile stula was made in the common bile duct. The bile flow was ry slow, only 0.1 cc. being collected in 30 minutes. 5 cc. of 5 per cent solution of alkali blue were injected into the femoral in. Bile samples were collected during successive 30 minute riods. No dye was found in Sample 1 but subsequent samples re all intensely blue. The color was greatly increased by acid ldition. The properties of the bile were the same as those scribed for Dog 2. The bile flow became rapid at first and en again slow, as shown by the volumes of the successive le samples measuring 0.1, 0.2, 0.2, 0.7, 1.9, 0.8, 0.2, 0.2, 0.2, 0.2, d 0.2 cc., respectively.

Autopsy.—6 hours after the dye injection the animal was killed by bleed- g. The contents and the mucous membrane of the alimentary tract owed no blue color, even after acid addition. The submucous tissue the alimentary tract, especially of the stomach, had a pale blue color, ough its alcohol extract did not show any blue tone. Alcohol extracts various organs and tissues, such as kidneys, spleen, mucous membrane the alimentary tract, blood, and mediastinal and mesenteric lymph ands, failed to show blue color. Only the liver and the bile in the gall adder contained the dye in a large amount. No dye was found in the ine; but protein and casts were present.

Experiment VII.—To induce marked flow of urine, Dog 7, male, 14 kg., was made diabetic by phlorhizin. 8 cc. of 2.5 r cent solution of alkali blue were then injected into the jugu- r vein. The amount of the urine in 24 hours after the dye jection was 1.6 liters. In spite of this diuresis, no dye was iminated through the kidneys; but an alcohol extract of the ces showed the presence of the dye.

Experiment VIII.—Dog 8, male, 8.2 kg., was used to see to hat extent alkali blue, injected intravenously, will be recovered the feces. 8 cc. of 2.5 per cent alkali blue solution were in- cted into the jugular vein. In order to cause discharge of ces, magnesium sulfate was administered by a stomach sound and 20 hours respectively after the dye injection. The feces 24 hours after the injection contained about 8 per cent of the re; during the next 24 hours, about 5 per cent. On the 3rd y only a trace of the dye was found in the feces.

Experiment IX.—Dog 9, female, 9.5 kg., was used to see to what extent alkali blue, administered by mouth, will be recovered in the feces. 8 cc. of 2.5 per cent alkali blue solution were given by a stomach sound. To induce purgation, magnesium sulfate (25 and 10 gm.) was administered into the stomach, immediately and again 20 hours after the dye administration. The feces in 24 hours after the dye administration contained 58 per cent of the dye, given by mouth.

Experiments with White Rats.

The body weight of the rats varied from 170 to 310 gm. In Rats 1 and 2, 1 cc. of 1 per cent alkali blue solution, in Rats 3 and 4, 1.5 cc. of 2.5 per cent alkali blue solution were injected intraperitoneally. No change of the urine flow and no elimination of the dye were observed. The dye was always present in the feces. 30 hours after the dye injection, the animals were killed. The peritoneum, especially the omentum, and pleuræ were intensely blue in color, but no dye was found in solution in the cavities. The liver appeared greenish blue. Mesenteric and mediastinal lymph glands were also stained with the dye. In the blood and the remaining viscera the dye could not be demonstrated. The test for it was positive in the intestinal contents. In Rat 5, for contrast, 1 cc. of 1 per cent indigo carmine solution was injected intraperitoneally. The dye was eliminated very rapidly through the kidneys and no trace of it was found in the feces. 48 hours after the dye injection the animal was killed. The dye could nowhere be demonstrated. In Rats 6 and 7, phlorhizin (0.05 and 0.1 gm.) was injected subcutaneously, to induce marked flow of urine. 1 cc. of 25 per cent alkali blue solution was injected intraperitoneally. Though the urine flow was greater than with the control animal, the dye was not eliminated through the kidneys, but was present in the feces. In Rats 8 and 9, 1 cc. of 2.5 per cent alkali blue solution was injected subcutaneously. The dye test was negative in the urine, but positive in the feces. The blue color at the injection area was very strong even after 1 month. In Rats 10, 11, and 12, powdered alkali blue was administered by mouth three times a day—about 0.05 gm. at a time. The feces were blue, but the urine contained no dye. After 2 or 3 days the animals were killed. The dye was found in the alimentary tract only, the alcohol extracts of the liver, kidneys, spleen, and blood always yielding negative results.

Experiments with Rabbits.

Experiment I.—Rabbit 1, 1.9 kg. 2 cc. of 2.5 per cent alkali blue solution were injected into an ear vein.

TABLE V.

Blood.[*]

Time after the dye injection.	Blue color.	
	Before acidification.	After acidification.
hrs.		
1	–	++
2	–	+
3	–	+
4	–	–

[*] Samples were taken from an ear vein of the other side.

The *urine* contained neither dye nor protein. The dye test was positive in the *feces*. (Diarrhea was induced by a purgative.)

Experiment II.—Rabbit 2, 1.6 kg. 1.5 cc. of 2.5 per cent alkali blue solution were injected into the jugular vein under cocaine local anesthesia. The result was the same as in Rabbit 1, except that the dye could not be demonstrated in the blood 3 hours after injection.

DISCUSSION.

Passage of Alkali Blue from Blood into Lymph.—Alkali blue, injected intravenously, appears very rapidly in the lymph of the thoracic duct—in a leuko form within 10 minutes, in a free form a few minutes later (Dogs 2 and 3). The delayed appearance of the dye in Dog 1 may have been due to the use of morphine and atropine which affect the permeability of the cellular membranes. Although the dye appeared also in the cervical lymph, its concentration was much less than in the thoracic lymph (Dog 4). Phenoltetrachlorphthalein and indigo carmine, injected intravenously, appeared in the thoracic lymph within 10 minutes. The passage of the latter dye seemed to be much easier than that of the former (Dog 5).

Disappearance from the Circulation.—In the dog experiments, blood samples taken 3 or 4 hours after the intravenous injection of alkali blue (21 to 26 mg. per kilo), contained the dye (Dogs 1, 2, and 3), but a sample taken after 6 hours showed a negative result (Dog 6). In the rabbit experiments the dye disappeared from the circulation in 3 to 4 hours after its injection. Phenoltetrachlorphthalein, on the other hand, could not be demonstrated 3 hours after its injection. Indigo carmine was not detected in the circulation 70 minutes after its injection (Dog 5). This difference in the rapidity of passage of the three unlike dyes from the circulation is characteristic. Keith and his collaborators showed that vital red remains in the circulation for a long time. The time needed for elimination from the blood vessels thus varied markedly, according to the properties of the dyes.

Effect upon the Lymph Flow.—The rate of lymph flow was not markedly changed after the alkali blue or phenoltetrachlorphthalein administration (Dogs 1, 2, 3, and 5). On the contrary, indigo carmine or physiological saline solution caused a rapid lymph flow (Dogs 1, 3, and 5).

Elimination in the Bile.—Evidence was obtained that the liver is the chief if not the only organ for the elimination of alkali blue. Alkali blue, injected parenterally (intravenously, intraperitoneally, or subcutaneously), was always found abundantly in the liver and in the bile. About 30 minutes after intravenous injection of alkali blue, the dye began to appear in the bile (Dog 6).

Effect upon the Bile Flow.—As seen in Dog 6, the bile flow increased markedly after the alkali blue injection, the maximum being 2½ hours after the dye injection. Though the effect of the narcotics or other factors may be of significance here, it is not improbable that the dye, which is chiefly eliminated into the bile, may of itself influence the rate of biliary flow. Abel and Rowntree, who investigated the effect of phenoltetrachlorphthalein upon the bile flow in an anesthetized dog, noticed no marked influence, the variations in rate being inconstant.

Elimination of Dye in the Urine.—In none of the experiments were more than traces of alkali blue eliminated in the urine. In some experiments with dogs a decrease of the urine flow was noticed. Protein and casts were also found. Ordinary diuretics were not effective. In addition to a possible toxicity the colloidal condition of the dye may have had some relation to the decrease of the urine flow, as Spiro and Buglia reported for gelatin or gum arabic. Even in the phlorhizinized animals (Dog 7, and Rats 6 and 7), which showed a profuse flow of urine, the dye was also absent from the urine. Indigo carmine, on the other hand, was always found in the urine in a very high concentration (Dog 5 and Rat 5). As alkali blue is a derivative of aniline the *p*-aminophenol test was performed with the urine. The results were always negative. Rosaniline (rosaniline trisulfate of soda) has been used as a functional test of kidneys by Lépine, Dreyfus, and others. It is very easily eliminated through the kidneys. The difference in behavior between rosaniline and alkali blue is somewhat similar to the difference between phenolsulfonephthalein and phenoltetrachlorphthalein.

Absorption and Elimination from the Alimentary Tract.—Experiments with Rats 10, 11, and 12 showed that alkali blue is not absorbed from the alimentary tract. Abel and Rowntree showed that phenoltetrachlorphthalein and phenolsulfonephthalein, eliminated in the bile, could be reabsorbed from the alimentary tract, the former from the large intestine, the latter from the whole intestine. No evidence similar to this was gained with the colloidal alkali blue. When alkali blue was administered by mouth and a purgative was given afterwards, 58 per cent of the dye was detected in the feces (Dog 9). But, when the dye was injected intravenously, its recovery in the feces was not so successful as has been reported for phenoltetrachlorphthalein (Rowntree's test for liver function). Part of the alkali blue seems to be changed into some undetected forms in the tissues and in the alimentary tract. This was also demonstrated *in vitro*. A mixture of the dye and feces emulsion loses its dye content gradually. Alkali blue was not eliminated from the mucous membrane of the alimentary tract, as shown in Dog 6, in which the bile was prevented from pouring into the intestine.

Absorption from the Subcutaneous Tissues.—Part of the alkali blue injected subcutaneously remained for a long time under the skin. In Rats 8 and 9 the area of the injection was intensely blue even after a month. In this respect, alkali blue is much different from phenoltetrachlorphthalein, which Abel and Rowntree could not find at the site of injection 16 to 24 hours after a subcutaneous administration.

SUMMARY.

Alkali blue, injected intravenously, rapidly appears in the lymph in both leuko and free states. In the cervical lymph it is less concentrated than in the thoracic duct lymph. The lymph flow is not affected markedly by the dye injection. Alkali blue, injected intravenously in doses of 21 to 26 mg. per kilo, remains in the circulation for a few hours only.

When administered parenterally, alkali blue is chiefly eliminated in the bile. The dye seems to act somewhat as a cholagogue. It is not eliminated by the kidneys except in traces. No evidence of its elimination or reabsorption through the alimentary tract was gained. When administered by mouth, the dye is not absorbed and more than a half of the given amount may be recovered in the feces with methods at present available. After intravenous injection the recovery of the dye in the feces is not so successful as has been reported for the somewhat comparably functioning phenoltetrachlorphthalein. The elimination of alkali blue through the liver seems to be rather slow and part of the dye is changed into some as yet undetected form in the alimentary tract. Absorption of the dye from connective tissue spaces is very tardy. These facts correspond with what might now be expected with a dye of the colloidal properties noted.

I desire to express my thanks to Professor Lafayette B. Mendel for his suggestions and criticism, also to Professor Frank P. Underhill for his help.

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EXPERIMENTAL STUDIES ON GROWTH.

VIII. THE INFLUENCE OF A DIET DEFICIENT IN FATS, AND OF THE SAME DIET WITH CHOLESTEROL ADDED, UPON THE GROWTH OF THE WHITE MOUSE.

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The experiments which are about to be described were originally undertaken with a view to determining the effect upon growth of a diet, otherwise as normal as possible, which is practically cholesterol-free. Such a diet, however, if composed of natural foodstuffs, was found to be necessarily almost lipoid-free, so that the effects observed may be, and in view of the failure of addition of cholesterol to neutralize these effects, probably are, attributable to the absence of other lipoids, or substances commonly associated with lipoids. The experiment therefore failed to accomplish its immediate purpose and the results obtained, as will be seen, merely afford a confirmation of the results previously reported by a number of investigators.[1] Since this confirmation was reached from a widely divergent angle, however, it appears to be worth reporting as affording a wider foundation for the view of the necessity of lipoids in the diet which has found general acceptance in consequence of the observations cited above.

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[1] Stepp, W., *Biochem. Z.*, 1909, xxii, 452; *Z. Biol.*, 1912, lvii, 135; 1913, lxii, 405. Mendel, L. B., and Osborne, T. B., *J. Biol. Chem.*, 1912, xii, 81; 1912–1913, xiii, 233; 1913–1914, xvi, 423. McCollum, E. V., Halpin, J. G., and Drescher, A. H., *ibid.*, 1912–13, xiii, 219. McCollum, E. V., and Davis, M., *ibid.*, 1913, xv, 167. Funk, C., *J. Physiol.*, 1911–12, xliii, 395; 1912, xliv, 50. Cooper, E. A., *Biochem. J.*, 1914, viii, 250. Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425. MacArthur, C. G., and Luckett, C. L., *J. Biol. Chem.*, 1915, xx, 161.

The diet administered consisted of boiled, peeled, and mashed potato, mixed for roughage with a small proportion of bran which had been defatted by extraction for 24 hours with hot alcohol followed by extraction for 24 hours with ether. This item of the diet was supplied *ad libitum*, water was also supplied *ad libitum*, and in addition to these items every group of six mice received daily (except Sundays) 10 cc. of white of egg to which were added five drops of Merck's water-soluble chlorophyll containing 0.2 per cent ferric chloride, equivalent to about 0.1 mg. of ferric chloride per mouse per day.[2]

In the case of the mice receiving cholesterol, 0.5 gm. of Merck's crystalline cholesterol was rubbed up with the 10 cc. of white of egg supplied to each six mice.

The diet of the animals which did not receive the added cholesterol was cholesterol-free, since Ellis and Gardner have found that neither potato nor white of egg contain any trace of cholesterol.[3] On the other hand, however, the diet was also very nearly fat-free, since white of egg contains only traces of fats, soaps, or phospholipins[4] and potatoes boiled in their skins contain only 0.03 per cent of fat.[5]

In other respects the treatment and manipulation of the mice were identical with the procedure previously reported.[6] Twenty-four mice of each sex and about 4 or 5 weeks of age were taken at random[7] and fed thereafter with the lipin-deficient diet. A similar number of males were similarly selected and fed thereafter with the lipin-poor diet plus cholesterol. The results are summarized in Tables I, II, and III and depicted graphically in Figs. 1, 2, and 3. In computing the average weights and riabilities the weights for 5 weeks preceding the death of any ividual were rejected excepting after the 28th week.

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[2] A small number of the mice did not receive the chlorophyll and iron for several weeks. No difference due to this omission was observed in the symptoms displayed.

[3] Ellis, G. W., and Gardner, J. A., *Proc. Roy. Soc., Series B*, **1913**, **lxxxvi**, 14.

[4] Hammarsten, O., Text-book of Physiological Chemistry, 6th edition, New York, 1911, 601.

[5] Williams, K. J., *J. Chem. Soc.*, 1892, lxi, 239.

[6] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, **1916**, **xxiv**, **347**.

[7] In replacing animals which died within 1 or 2 weeks of initiating the diet large animals were purposely selected. Hence the initial average weight of the survivors was, in each experimental group, supernormal.

TABLE I.

le Mice Fed on Fat-Deficient Diet. Mean Duration of Life 27.2 Weeks. Variability of Duration of Life 37 Per Cent.

Age.	Weight.		No. weighed (fat-deficient diet).
	Normal.	Fat-deficient diet.	
wks.	*gm.*	*gm.*	
4	12.38	15.29	9
5	12.45	14.31	16
6	15.58	14.41	17
7	18.08	15.09	17
8	19.36	15.00	15
9	20.63	15.17	15
10	21.19	16.08	13
11	21.81	16.58	13
12	22.65	16.73	13
13	23.31	17.27	13
14	23.96	18.29	12
15	24.28	18.96	12
16	24.75	19.13	12
17	25.21	20.64	11
18	25.61	21.50	11
19	25.81	21.91	11
20	26.10	21.91	11
21	26.28	21.68	11
22	26.06	22.15	10
23	26.34	21.70	10
24	26.82	21.40	10
25	27.05	20.85	10
26	26.94	20.65	10
27	26.55	20.15	10
28	27.19	19.80	10
29	27.08	18.55	10
30	27.23	22.33*	6

* The apparent rise in weight at this point is due to the lightest animals ving succumbed.

From the first the animals failed to do well on this diet. A ge mortality occurred during the first few weeks and a majority the animals were afflicted with diarrhea during this period. is rendered it a very difficult matter to distinguish with cer-

TABLE II.

Female Mice Fed on Fat-Deficient Diet. Mean Duration of Life 27.8 Week
Variability of Duration of Life 33 Per Cent.

Age.	Weight.		No. weighed (fat-deficient diet).
	Normal.	Fat-deficient diet.	
wks.	*gm.*	*gm.*	
4	10.39	13.17	6
5	11.81	12.03	17
6	14.12	11.80	22
7	16.77	12.38	20
8	17.99	12.85	20
9	18.78	12.70	20
10	19.38	13.22	18
11	20.04	13.79	17
12	20.31	13.59	17
13	21.04	13.94	17
14	21.21	14.38	17
15	21.78	15.41	17
16	22.14	15.68	17
17	22.29	16.06	17
18	22.22	16.74	17
19	22.60	17.03	17
20	22.60	17.30	15
21	23.06	17.07	15
22	23.32	17.21	14
23	23.51	17.18	14
24	23.68	17.15	13
25	23.79	16.73	13
26	24.04	16.35	13
27	24.00	16.35	13
28	23.58	15.85	13
29	23.84	15.14	11
30	23.92	15.60	10

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* Except the female which was fed after the 44th week upon tethel and which survived to the age of 64 weeks.

tainty the animals affected with β-paratyphoid while, on the other hand, had every animal displaying diarrhea been sacrificed, nearly all of the experimental material would have been lost. As an approximate method of evading this difficulty, blood-stained feces or exceptionally severe diarrhea were held indicative of β-paratyphoid, and animals displaying these symptoms

TABLE III.

e Mice Fed on Fat-Deficient Diet Plus Cholesterol. Mean Duration of Life 22.2 Weeks. Variability of Duration of Life 41 Per Cent.

Age.	Weight.		No. weighed (fat-deficient diet + cholesterol).
	Normal.	Fat-deficient diet + cholesterol.	
wks.	*gm.*	*gm.*	
4	12.38	11.42	6
5	12.45	12.96	14
6	15.58	13.45	21
7	18.08	14.10	20
8	19.36	15.00	17
9	20.63	15.53	15
10	21.19	15.46	13
11	21.81	15.65	13
12	22.65	16.54	12
13	23.31	16.67	12
14	23.96	17.04	12
15	24.28	18.42	12
16	24.75	20.05	11
17	25.21	19.95	11
18	25.61	19.27	11
19	25.81	19.70	10
20	26.10	20.64	7
21	26.28	20.50	7
22	26.06	19.79	7
23	26.34	21.00	6
24	26.82	20.58	6
25	27.05	20.08	6
26	26.94	20.00	6
27	26.55	19.16	6
28	27.19	19.00	6
29	27.08	18.50	6
30	27.23	18.08	6

killed and replaced by others, the cage and food and water
ainers being washed down with alcohol.

uring this period a decided loss of weight was suffered by the
ials receiving the lipin-poor diet alone. The animals re-
ing this diet with the addition of cholesterol, however, showed
oss of weight, save in a few individual cases, but, on the con-
y, a decided gain from the very first (compare Tables I and
It is difficult to interpret this very striking difference

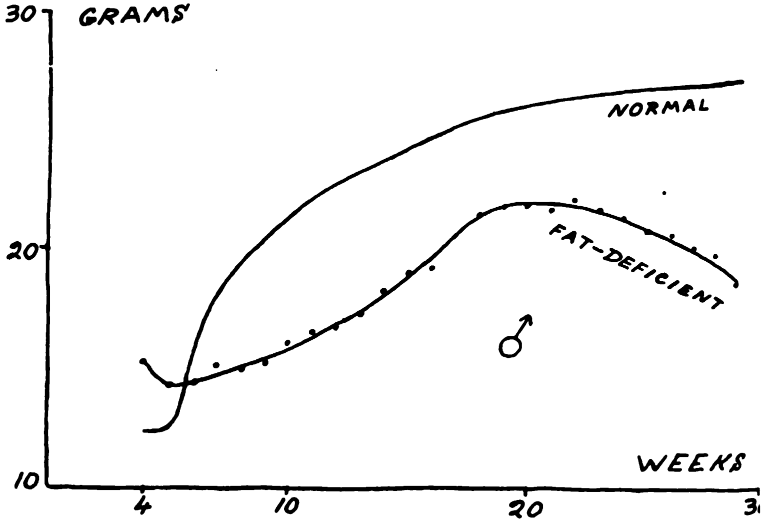

Fig. 1. Comparison of the growth curves of normal males and of male fed upon a diet deficient in fats.

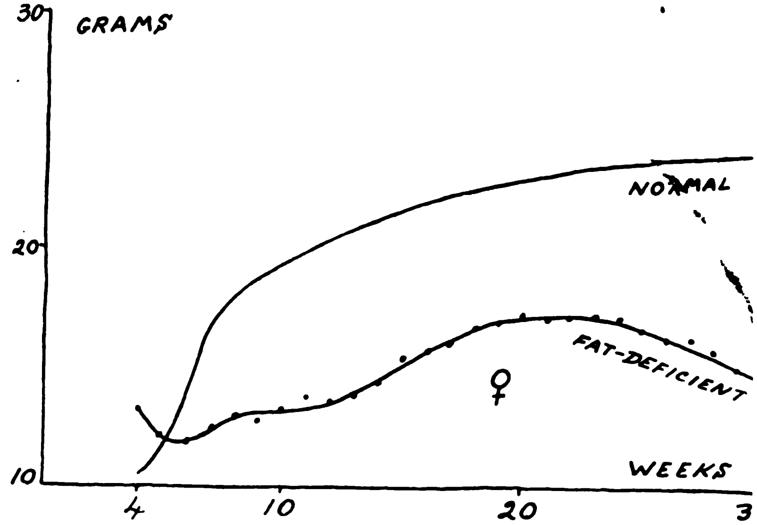

Fig. 2. Comparison of the growth curves of normal females and females fed upon a diet deficient in fats.

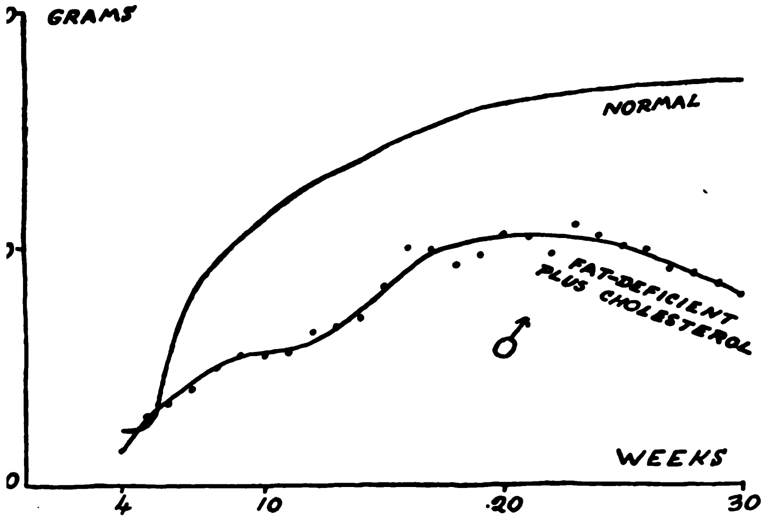

FIG. 3. Comparison of the growth curves of normal males and of males d upon a diet deficient in fats, to which cholesterol has been added.

etween the behavior of the two groups of animals, more especilly as the animals receiving cholesterol displayed no such supeority in the later weeks of the experiment and actually survived r a shorter period than the animals which did not receive olesterol.

In addition to diarrhea and loss of weight, the animals fed pon the lipin-free diet also displayed, during the first few weeks fter the initiation of the diet, the following symptoms: The ils were cold, clammy, and pale, and there was a decided tenency to develop gangrene in the tip of the tail, with resultant ss of several joints. The ears were perfectly white, the penis as usually extended and became inflamed and frequently ganrened. The rectum appeared inflamed. The movements of e animals were languid and they were notably lacking in rength. The animals receiving cholesterol also displayed ese symptoms, but in a milder degree.

At 8 or 9 weeks of age, that is, about a month or 5 weeks after e initiation of the diet, the condition of the animals noticeably improved although it was at no period remarkably good. They then gained in weight, the lesions in the tail and penis healed, and the appearance of the coat improved. A fairly steady although slow accretion of weight continued until about the 22nd week (a highly variable period in the different individuals) when a sudden and rapid loss of weight occurred, accompanied by marked hyperirritability of the skin resulting in incessant scratching and in a few cases in punctate effusion of blood at the surface of the skin. The movements became weak, tremulous, and uncertain. The animals ate incessantly, and in some cases the urine had a markedly ammoniacal odor. After a varying period, usually about 4 or 5 weeks, the animals died, sometimes after several convulsions of brief duration followed by apparent recovery. The animals receiving cholesterol showed no superiority over the animals receiving the lipin-free diet alone, and their duration of life was, as will be seen, even briefer. It is a fact which is perhaps not devoid of significance that the final rapid loss of weight and incidence of death occurred in each group of animals at an average age approximately coinciding with a period of relative instability in the growth of normal animals.[8]

The duration of life in each group was highly variable and some individuals even survived for over 40 weeks. One of these individuals (a female) at 44 weeks had already lost 2 gm. in the preceding month and by analogy with the other animals had only a few days to live. 4 mg. per day of tethelin were now administered to this animal, with the white of egg. The lost weight was slowly regained during the succeeding month and this animal attained the age of 64 weeks. The usual sharp drop in weight and hyperirritability made their appearance at the 60th week, however, and the symptoms preceding death did not differ in any noticeable fashion from those displayed by the other animals. Whether the great prolongation of life in this individual, as compared with the other members of its group, was attributable to the administration of tethelin or not, cannot of course be decided without more extended investigation.

From these results it is evident that although growth is possible n the diet described, it is impossible to maintain beyond a ertain period the tissue which has been gained. In fact, paraoxical as it may appear, we have here a diet upon which, in ontrast to certain diets lacking particular amino-acids,[9] growth possible but maintenance is not.

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[8] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 372.

The influence of these diets upon the variability of the animals o which they were fed is summarized in Table IV. In each

TABLE IV.

Variability.

Age.	Males.			Females.	
	Normal.	Fat-deficient diet.	Fat-deficient diet + cholesterol.	Normal.	Fat-deficient diet.
wks.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
4	24.4	19.6	14.2	23.4	12.0
5	24.6	18.2	19.8	19.4	16.0
6	22.0	20.3	16.6	18.9	15.4
7	16.9	21.8	20.1	15.0	16.4
8	15.9	22.9	19.6	13.9	17.9
9	16.5	24.9	18.2	13.4	20.9
10	16.7	21.3	20.7	14.3	20.2
11	13.3	23.6	20.8	12.6	23.4
12	13.7	21.3	21.9	12.6	18.2
13	14.2	23.3	20.3	13.0	20.2
14	14.1	23.9	20.9	13.2	19.9
15	11.9	22.8	21.6	13.7	22.6
16	12.7	22.5	15.2	12.6	22.1
17	12.4	19.9	15.0	11.9	22.7
18	12.1	20.8	15.9	11.9	22.5
19	12.1	19.7	19.2	12.0	23.7
20	10.8	17.8	19.4	11.2	22.7
21	11.2	18.9	20.3	12.3	23.4
22	9.0	18.3	21.3	12.5	18.7
23	9.8	20.3	13.6	12.0	19.3
24	10.1	20.4	13.7	11.3	22.9
25	11.0	20.8	17.1	11.4	21.9
26	10.8	23.0	18.1	11.6	22.1
27	11.3	24.6	19.8	11.8	23.1
28	10.9	27.0	20.4	10.3	24.0
29	10.9	29.2	20.1	12.2	26.5
30	9.5	14.4	25.7	12.4	27.4

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[9] Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325.

instance the initial variability of the animals was subnormal. Yet in a very brief period of feeding upon the diets the variability became supernormal and instead of decreasing with slackening growth, as in the normals, maintained a high level throughout. This would probably be found to be a general effect of adverse conditions of life, since unfavorable dietetic or environmental conditions might be expected to enhance preexisting deviations from the mean, initially supernormal animals resisting the pressure of the unfavorable environment more successfully than the average and thus departing more widely than before from the now subnormal average, while initially subnormal animals may be expected to become more subnormal than ever.

SUMMARY.

1. A diet composed of boiled and mashed potatoes, defatted bran, and white of egg, with the addition of small amounts of chlorophyll and ferric chloride, when fed to mice of 4 or 5 weeks of age, leads to initial loss of weight followed by resumption of a retarded growth. Ultimately, however, a sharp decline in weight occurs, accompanied by marked hyperirritability of the skin and terminated by death.

2. The addition of cholesterol to the above diet prevents the initial loss of weight, but does not otherwise improve the welfare of the animals, the average duration of life of the animals receiving cholesterol being actually less than that of the animals which do not receive cholesterol.

3. It follows that although growth is possible on the diet described, maintenance of the tissue gained is not. Decline of weight therefore occurs at an age when the diminishing velocity of growth has become insufficient to compensate for the deficient ability of the tissues to replace their current loss.

4. The variability of animals fed upon the diets described, as is probably to be expected under any unfavorable environmental or dietetic conditions, instead of falling with increasing age and slackening of growth, maintains a high level throughout the life of the animals.

SIOLOGICAL AND PHARMACOLOGICAL STUDIES ON COAL TAR COLORS.

I. EXPERIMENTS WITH FAT-SOLUBLE DYES.

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though synthetic dyes have played an important part in study of biology and medicine, the behavior of many of : compounds in the body is still imperfectly understood the action of some of them is entirely unknown. Owing to extensive employment in numerous industries, and espe- r in the preparation of foods, this lack of satisfactory in- ation frequently proved to be a matter of serious import estions regarding their effect on health were often raised, o definite answer could be given in the present state of our dedge.

e recognition of the need of a more intimate acquaintance the reactions produced in the body by these compounds ed the starting point of various experimental inquiries in laboratory, the present report being the first of a series mmunications embodying the results of some of these tigations.

evious observations on the changes which might occur in luble dyes within the body or on the effects produced by . have been confined to only a few members of this group were limited in scope. The experiments reported dur- he past two decades by Daddi (1), Biedermann (2), Sit- ki (3), Hofbauer (4), Riddle (5), S. H. and S. P. Gage (6, Iann (8), and others dealt chiefly with selective action and ionally with the path of elimination, benzeneazobenzene- -naphthol (Sudan III) being largely employed in these es. But the more recent investigations on this subject, notably those of Mendel and Daniels (9), included attempts to gain information concerning the mode of absorption, distribution, elimination, and also toxicity which were carried out with a number of fat-soluble dyes, including Sudan III.

The present report embraces the results of experiments with fat-soluble dyes which were made on different animals. Although the fate of these compounds in the body was the main object of the investigation, observations were also made on toxicity. The following compounds were employed, the commercial names most commonly used and their numbers in Schultz's tables are given at the right.

Benzeneazo-β-naphthylamine	Yellow A.B.**
Tolueneazo-β-naphthylamine	Yellow O.B.**
Benzeneazobenzeneazo-β-naphthol	Sudan III 223*
Benzeneazo-β-naphthol	Sudan I 36*
Benzeneazodimethylaniline	Butter Yellow 32*
Benzeneazophenol	Oil Yellow.
Benzeneazoresorcinol	Sudan G 35*
Aminoazobenzene	Spirit Yellow 31*

* Schultz, Farbstofftabellen, Berlin, 1914.

** Heller and Mertz.

Rabbits were mainly used as subjects of the experiments but observations with some dyes were also made on a few cats and on rats.

Methods.

The dyes were administered subcutaneously, intraperitoneally, intravenously, and by mouth. For subcutaneous and intraperitoneal injections they were dissolved in cottonseed oil. In a few experiments at the beginning of the research, liquid gelatin or starch suspensions were also employed for this purpose in order to avoid a possible lipuria. Intravenous injections were given in 1 per cent sodium hydroxide or in emulsions made up with 5 per cent acacia, cottonseed oil, and sodium carbonate, the amount of dye in both cases being 0.25 per cent. All the dyes employed in these experiments were carefully purified before using since it had been shown by some investigators (10) that impurities caused a marked increase in their toxicity. In feeding experiments which were carried out on rats the dye was incorporated with the food. Inquiries were also conducted to determine the effect of renal disturbance on the elimination of fat-soluble dyes in the urine. Zinc salts were mixed with the food and fed to rats with the dyes. Zinc malate was also introduced intravenously into rabbits, while the dye was given subcutaneously, a short space of time intervening between the two operations. Experiments were also made on the effect of oil of chenopodium on the elimination of dyes. The presence of dye in the urine and bile was determined by inspection and by dyeing wool.

I. The Elimination of Dyes in the Urine.

Whatever the mode of administration employed in our experiments, the presence of the different dyes in the urine (as well as in the bile) could be observed in every case in normal animals.

Their appearance in the urine could be observed within ½ to 2 hours after they were injected subcutaneously. Elimination usually continued several days and sometimes extended over several weeks even when only moderate doses were administered. Thus after the administration of 0.25 gm. per kilo of benzene- or tolueneazo-β-naphthylamine the dyes were present in the urine for 16 to 17 days. When 0.7 and 0.77 gm. per kilo of aminoazobenzene were given to two rabbits respectively the dye was still present in the urine at the end of 20 days. After injecting 0.6 gm. per kilo of benzeneazodimethylamine the urine was free from dye only at the end of 22 days. In experiments with benzeneazoresorcinol dye was still present 8 to 13 days after doses of 0.2 to 0.3 gm. per kilo were given. When 0.5 and 0.65 gm. of it per kilo were injected into two rabbits dye could be observed in the urine for 60 days. The rate of elimination by the kidney was much faster after intravenous injections. In most experiments the dyes appeared in the urine within 9 to 36 minutes. In some cases their appearance was delayed much longer. In one experiment it was first noticed in the urine after 80 minutes. The size of the dose seemed to have a marked influence on the speed of elimination, as was found in experiments with benzeneazoresorcinol. When about 100 mg. of the dye per kilo were given elimination lasted 1 to 3 days, while doses of 25 to 36 mg. per kilo disappeared from the urine at the end of 2 to 6 hours. In two experiments, however, with 6 and 8 mg. per kilo elimination lasted 3 to 4 hours. It may be remarked that the medium in which the dye was administered was without any effect on the rate of elimination, the speed being the same whether given in the form of an emulsion or dissolved in sodium hydroxide.

That the different dyes employed (in these experiments) are also eliminated in the urine when they are given by mouth was shown in a number of experiments. Two cats received 100 mg. per kilo of benzeneazoresorcinol with their food. The next morning, that is, in about 17 hours, the urine was colored with dye. Elimination ceased in 24 to 48 hours. Similar results were obtained with other fat-soluble dyes that were fed to different animals. Rats that had been receiving daily with their food 60 to 70 mg. of benzeneazo-β-naphthylamine or 120 to 140 mg. per kilo of body weight of the toluene derivative eliminated these compounds in the urine; this was also observed in two rabbits that were given 1 gm. per kilo of these dyes by mouth. Elimination in the urine was likewise observed after intraperitoneal injections of these dyes when suspended in gelatin or starch as well as when dissolved in cottonseed oil. As tests for the presence of fat in the urine after the administration of the dyes were invariably negative, the observation that these substances are eliminated by the kidney suggested the advisability of ascertaining in what form they were present in the urine.

It was shown long ago by Schmiedeberg (11) that aniline given to animals is partly eliminated as glucuronate and partly as sulfate. It has also been established that various aniline derivatives undergo similar changes in the body, as shown in the case of antipyrin, acetanilide, and acetophenetidine. According to Gautrelet and Gravellat (12), marine blue is eliminated by the kidney in combination with sulfuric acid.

Conjugation with sulfuric or glucuronic acid was naturally thought of as the mechanism by which the various dyes which we have studied might be transformed into water-soluble compounds. This suggestion was tested in two dyes, benzeneazoresorcinol and benzeneazophenol, the following procedure being adopted.

The Separation of the Glucuronate of Benzeneazoresorcinol.

The urine of rabbits which had received the dye was treated with immiscible organic solvents such as amyl alcohol, ethyl acetate, benzene, ether, toluene, and chloroform, but all the attempts to extract it proved unsuccessful. Other procedures for the isolation of the substance were therefore resorted to. It was found that on treating the urine with dilute hydrochloric acid the dye could be precipitated. Care must be taken to avoid an excess of the acid as the precipitate may be redissolved if this precaution is not observed. After stirring thoroughly and cooling with ice, the mixture was allowed to stand several hours and then filtered by suction. The filtrate was still dark colored although no further precipitation could be obtained with acid. The brownish red precipitate was washed with cold water a few times and then crystallized from dilute alcohol. The substance crystallized in yellow to pale brown needle-shaped prisms melting at 189–190°C. with decomposition, the yellow substance changing to a red color at 130–140°C. without undergoing any other apparent transformation.

The nitrogen determinations were made by the modified Kjeldahl method of Phelps and Daudt (13). The substance was dried at 115°C. for 6 hours. The calculated amount of nitrogen in glucuronate of benzeneazoresorcinol, $C_{18}H_{20}N_2O_9$, was 6.86 per cent; found, 6.85 per cent.

Reactions of Glucuronate of Benzeneazoresorcinol.—The substance dyed wool yellow, gave no test for sulfur or halogen, and did not respond to the resorcinol test. On reduction in aqueous or alcoholic solution with stannous chloride the yellow color disappeared and did not return when exposed to the air, even when treated with oxidizing agents.

Hydrolysis.—0.5 gm. of the glucuronate was suspended in 100 cc. of water to which 2.5 cc. of HCl (sp. gr. 1.19) were added. Hydrolysis was effected by boiling the mixture until the solution became red. When boiled for 1 hour a red substance separated out on cooling. It was made slightly alkaline with sodium hydroxide and warmed on the steam bath for 1.5 hours, the clear red solution cooled, and then neutralized with acetic acid. A red precipitate was obtained which was filtered off. This substance was insoluble in water and crystallized from dilute alcohol in beautiful acicular crystals which melted at 165°C. to a clear oil. When mixed with benzeneazoresorcinol (melting point 167–8°C. to an oil) the mixture also melted at 167–8° C. to a clear oil.

Furfurol-HCl Distillation.—0.5 gm. of the substance was suspended in HCl (sp. gr. 1.06) and distilled according to the method described by Abderhalden (14). The distillate gave a strong test with aniline acetate and on treatment with phloroglucinol the solution turned bright yellow, then green, and finally became turbid with a greenish black precipitate of the furfurol-phloroglucid.

Naphthoresorcinol Reaction.—The conjugated product was boiled in 10 cc. of water containing a few drops of concentrated HCl, cooled, and treated with an equal volume of concentrated HCl. (The substance would not respond to the test without this previous hydrolysis.) To this were added 2 cc. of an alcoholic solution of naphthoresorcinol and the mixture was boiled for 1 minute, allowed to cool, and extracted with ether. The ether took on a violet color and showed an absorption band at D.

The Separation of the Glucuronate of Benzeneazophenol.

The urine containing this substance behaved towards immiscible extracting solvents in a manner similar to the one previously described. The 24 and 48 hour urines of rabbits which had received benzeneazophenol were treated with concentrated HCl until precipitation was complete. The mixture was cooled with ice and the yellow precipitate filtered off by suction. This product was crystallized from dilute alcohol from which it separated in yellow rectangular plates or, sometimes, acicular prisms melting at 164–5°C. with decomposition.

The substance was dried at 110°C. for 4 hours. The amount of nitrogen calculated in benzeneazophenolglucuronate ($C_{18}H_{20}N_2O_8$) was 7.14 per cent; found, 6.85 and 6.76 per cent.

Reactions of the Glucuronate of Benzeneazophenol.—The substance dyed wool a light yellow, gave no test for sulfur or halogen, and did not respond to the Millon test. It was reduced by tin chloride in aqueous or alcoholic solution to a colorless solution but the color did not return on oxidation.

Hydrolysis.—0.15 gm. of the glucuronate was suspended in 25 cc. of water and treated with 0.6 cc. of concentrated HCl. The mixture was boiled gently under a reflux condenser, whereupon the substance gradually went into a light red solution with a floating dark red oil. The hydrolysis was continued for 4 hours and on cooling, yellow prismatic crystals separated. This substance was filtered off and washed with cold water. On crystallization from dilute alcohol peculiar hairy crystals were deposited which melted at 154–5°C. to a dark red oil. These crystals gave a Millon test and on mixing with some benzeneazophenol, which melts at 154–5°C. to an oil, the mixture also melted at the same temperature.

Furfurol-HCl Distillation.—65 mg. of the glucuronate were suspended in HCl (sp. gr. 1.06) and distilled as previously described. The distillate gave a strong Schiff test with aniline acetate and also a characteristic furfurol reaction with phloroglucinol.

Naphthoresorcinol Test.—This test was carried out exactly as described under the glucuronate of benzeneazoresorcinol. A positive reaction was obtained and the violet-colored ether showed an absorption band in the yellow at D.

On account of the color imparted to a solution of these glucuronates and the low specific rotation of glucuronic acid, no very satisfactory results could be obtained as regards optical activity. 1 per cent solution of the phenol derivative in 50 per cent alcohol showed a rotation of $-3°$ in a 0.5 dm. tube. After hydrolysis with dilute sulfuric acid this rotation was decreased to 0° but because of the energetic treatment which is necessary for the hydrolysis of this product, the glucuronic acid was probably decomposed and no positive rotation of the free acid could be detected. In another experiment where less material was utilized, the hydrolysis changed the rotation from -0.3 to $+0.1°$. However, these readings in dark colored fields are within the limits of experimental error. The synthesis with glucuronic acid, as established in the case of benzeneazophenol and benzeneazoresorcinol, indicates that the behavior of these substances in the body is similar to that of other members of the aromatic series. It may be inferred, therefore, that the other fat-soluble dyes whose elimination in the urine was observed had undergone a similar change, those not possessing a hydroxyl group first being oxidized to hydroxy derivatives.

II. The Elimination of Dyes in Diseases of the Kidney.

In a number of experiments on rabbits that received intravenously 10 mg. of zinc per kilo in the form of malate, marked albuminuria was present, severe lesion of the kidney being found on microscopic examination. The elimination of dyes which were given subcutaneously was very slight at first. In about 21 to 24 hours they completely disappeared from the urine.

As mentioned above, the elimination of fat-soluble dyes was observed in rats when these substances were incorporated with the lard that formed one of the ingredients of their diet. In two of these experiments zinc was given with the food, 40 gm. of which contained 60 mg. in the form of the acetate or of the sulfate. The urine examined 18 hours later failed to show the presence of the dye, thus indicating that renal function may be impaired even when the metal is given with the food.

Evidence of decreased permeability of the kidney was also present after the subcutaneous injection of oil of chenopodium, but the results were not constant, elimination being inhibited in only eight out of fourteen rabbits. Almost complete suppression of elimination was observed in one experiment in which the dyes were injected several days previous to the administration of oil of chenopodium. In two others, in which it was given immediately before the oil of chenopodium, the same results were obtained, none being eliminated during the first 24 hours, while the urine obtained on the following day was but slightly colored. In a third series of experiments, typical protocols of which are given below, the evidence of decreased permeability of the kidney after the administration of fat-soluble dyes was even more satisfactory.

Rabbit 1,671. Belgian Male. Weight 1,395 Gm. Diet, Oats.

Jan. 18. Subcutaneous injection of 0.75 gm. of benzeneazodimethylaniline dissolved in 15 cc. of cottonseed oil.

Jan. 19, 9 a.m. Urine collected. Contained considerable amounts of dye. 1 p.m. 0.55 cc. oil of chenopodium given by mouth in 15 cc. of cocoanut oil.

Jan. 20, 9 a.m. Rabbit found dead. The urine which passed since the oil of chenopodium was fed did not contain any dye. This rabbit had albuminuria for the last 6 days.

Rabbit 1,694. Black Male. Weight 1,930 Gm. Diet, Oats.

Jan. 21. Subcutaneous injection of 0.5 gm. of benzeneazophenol in 10 cc. of bleached cottonseed oil.

Jan. 22, 9 a.m. Urine collected. Contained a considerable amount of dye. 2.47 p.m. 0.8 cc. of oil of chenopodium in cocoanut oil given by mouth. Elimination of dye continued unchanged for the next 3 days, but the elimination was permanently suppressed after this.

Rabbit 1,665. Belgian Female. Weight 1,400 Gm. Diet, Oats.

Jan. 7, 11.10 a.m. Subcutaneous injection of 1 gm. of benzeneazophenol in bleached cottonseed oil. 4.10 p.m. Urine obtained from bladder showed presence of dye.

Jan. 11. Dye still eliminated in urine, 0.56 cc. of oil of chenopodium in 15 cc. of cocoanut oil given by mouth.

Jan. 12. Urine showed the presence of slight amounts of dye.

Jan. 13. Urine did not contain any dye. An examination of the urine daily for several days failed to indicate the presence of dye.

The experiment was repeated on another rabbit with the same result. In both cases albumin was found in the urine on the 3rd day after the injection of chenopodium and persisted for several weeks. In some rabbits, however, the elimination of dye continued uninterruptedly after oil of chenopodium was given although albumin was present in the urine. Permeability of the kidney for the dyes studied may be independent, therefore, of albuminuria which is frequently physiological in the rabbit (15). The failure of the dye to pass out in the urine could, nevertheless, be attributed to renal disturbance. It has been shown by Salant and Livingston (16), that oil of chenopodium produced a marked fall of blood pressure with a pronounced decrease in the volume of the kidney. Impaired function due to deficient nutrition caused by the lessened blood supply might thus interfere with the eliminating power of the kidney. This would seem to be highly probable in view of the experiments of Litten (17), who observed different degrees of injury after clamping the renal artery for 1½ to 2 hours. On the other hand, abolition of the synthetic function of the liver might be regarded as the cause of the absence of the dye from the urine in poisoning with oil of chenopodium. That this was not the case appeared from experiments which were carried out on rabbits with hydrazine, which is a hepatic poison. Oil-soluble dyes given to such animals were eliminated in the urine as was observed in the case of normal animals.

III. The Elimination of Dyes in the Bile.

Abel and Rowntree (18) observed that the oil-soluble tetrachlorophenolphthalein is eliminated in the bile but not in the urine. The experiments of Mendel and Daniels (9) with a number of fat-soluble dyes showed that the bile was the sole path of elimination for all those compounds which they examined. As shown in the preceding pages this view cannot be supported in the light of our observations on the subject. Their statement that these dyes may be eliminated by the liver, however, was fully corroborated by us in the present investigation. Examination of the bile, made after a proper interval following the administration of the different dyes which we have studied, showed that the liver played an important part in the elimination of these compounds. Bile removed from the gall bladder of a rabbit 14 days after the subcutaneous injection of tolueneazo-β-naphthylamine contained a small amount of the dye. Evidence of elimination by this path was also obtained in experiments with benzeneazo-β-naphthol. The bile of three rabbits which received 0.5 to 0.6 gm. per kilo subcutaneously was still deeply colored at the end of 12, 13, and 18 days. The results were more striking after intravenous injections, the bile being obtained from a temporary fistula. In experiments on three rabbits which received 0.022, 0.029, and 0.043 gm. per kilo intravenously, the color was noticed in the bile in 16, 17, and 18 minutes respectively. Similar results were obtained in experiments with several other dyes. The period of elimination was found to be relatively short. After the intravenous injection of 0.130, 0.140, and 0.160 gm. of benzeneazoresorcinol per kilo, no dye could be observed in the bile at the end of 4 hours. It may be remarked that the period of elimination when a dose of 0.020 gm. per kilo of benzeneazophenol was injected intravenously was 3½ hours. The probable significance of this will be discussed later. It is interesting to observe that elimination in the urine continued for some time after the dyes disappeared from the bile. After the subcutaneous injection of benzeneazoresorcinol the bile in two experiments was free from dye at the end of 8 and 13 days but it was still present in the urine. This was also noticed after intravenous injections. A typical illustration of this is presented in the following experiment.

Rabbit 2,023.

9.45 a.m. Ether administered, cannula inserted in bile duct, and wound closed.

10.10. Bile light green.

10.21. 5 cc. of benzeneazoresorcinol in 1 per cent sodium hydroxide, injected into ear vein in 6 minutes.

10.36. Dye appeared in urine.

11.10. Urine obtained from bladder normal in appearance.

11.25. Urine very faintly tinged with dye.

11.40. Presence of dye in urine distinct.

1.30 p.m. 13 cc. of reddish brown bile collected. Bile light colored and became normal in appearance in about 1 hour.

4.15. 5 cc. of bile, yellowish green.

The following day, 9 a.m., 23 cc. of olive-green bile collected.

12.20 p.m. Urine obtained from bladder still colored with dye. Rabbit chloroformed.

The observation in this, as in several other experiments, that the dye continues to pass out in the urine after its disappearance from the bile is very suggestive, as it points to the kidney and not to the liver as being the chief organ of excretion for these compounds. This is particularly interesting in view of the results of Mendel and Daniels (9) who, it will be recalled, maintained that the fat-soluble dyes are eliminated entirely by the liver, their presence in the urine being observed only when lipuria existed. The decreased permeability of the kidney which we noticed in zinc nephritis suggests that a similar process might have been operative in the experiments of these investigators. Our observations on the inhibitory effect of oil of chenopodium on the elimination of dyes make this supposition highly probable for there was no evidence of renal injury caused by the oil, which indicates that the mechanism for the elimination of these substances might be deranged although no abnormality was observed in the appearance of the kidney or of the urine secreted. The discrepancy between the results of Mendel and Daniels and those which we obtained may thus be explained. It is entirely possible that the animals they used in their experiments were not normal.

IV. Are Dyes Eliminated into the Gastro-Intestinal Canal?

Observations have also been made on the elimination of fat-soluble dyes into the gastro-intestinal canal. In one experiment in which 0.29 gm. of benzeneazo-β-naphthol per kilo was given intravenously to a rabbit, the contents of the lower portions of the jejunum and of the ileum were stained but no evidence of the presence of dye in the rest of the gastro-intestinal canal could be obtained in this case. The experiment was repeated on another rabbit but no dye could be seen in any part of the gastro-intestinal canal. Tests with several other fat-soluble dyes used in this investigation likewise proved negative. The presence of the dye in the intestine might, therefore, be due to abnormal conditions. The recent report of Lewis (19) on trypan red is suggestive. After the intravenous injection of this substance into rabbits he observed large amounts of the dye in the aqueous humor when the eye was congested, but this fluid was free from color when the eye was normal.

V. The Distribution of Fat-Soluble Dyes in the Body.

Observations on this subject have been made by several investigators. Mann (8) claimed that animals fed oils colored with benzeneazobenzeneazo-β-naphthol show only staining of the adipose tissue. S. H. and S. P. Gage (7) failed to observe staining of the nerve fibers of chicks developed from eggs stained with benzeneazobenzeneazo-β-naphthol, but they reported that the adipose tissues were stained with the dye. Mendel and Daniels (9), who experimented with a number of fat-soluble dyes on different animals found that, with a few exceptions, the adipose tissue and bone marrow were stained. This was rarely the case with the muscles while the nervous system and kidneys appeared to be consistently free from dyes. Their presence in the liver could be observed in some cases but in the greater proportion of experiments no staining of this organ could be noticed after the administration of any of the coloring substances.

Our studies on the distribution of the different dyes in the body were made on rabbits and on cats. We found that the adipose tissues were always colored with the dye, no matter how introduced into the body, the color persisting even after its elimination in the bile and urine had ceased. Staining of the other tissues was seldom noticed. In a few instances only could any dye be seen in the nervous system. A cat which had fasted 6 days received intraperitoneally 2 gm. of benzeneazo-β-naphthylamine per kilo, suspended in starch solution. The urine was colored until the death of the animal which occurred 45 hours after the dye was administered. On autopsy the dye could be seen very distinctly in the brain, nerves, voluntary muscles, subcutaneous tissues, and in the fat. It may be observed that a large amount of dye, apparently unchanged, was still present in the peritoneal cavity. A second experiment with this dye was repeated on a well fed cat, 1.3 gm. per kilo suspended in starch being administered intraperitoneally. The cat died 45 hours later. The subcutaneous tissues and fat of the body were likewise colored in this case; neither the brain nor spinal cord showed the presence of dye but the nerves were stained. The same results were obtained with tolueneazo-β-naphthylamine dye, which was also administered intraperitoneally to a well fed cat, a dose of 0.88 gm. per kilo suspended in starch being given. The duration of life in this case was 9 days. The urine contained the dye during the life of the animal. A dose of 1.1 gm. per kilo was given to another cat in the same way. The duration of life was nearly 6 days. Slight coloration of the subcutaneous tissues was observed but the nervous system was free from dye.

That the duration of life might be an important factor in the persistence of the various dyes in the tissues was shown in the following experiments in which the substance was introduced directly into the circulation. In two rabbits in which benzeneazoresorcinol, 0.1 gm. per kilo, was given intravenously it was present in the subcutaneous tissues, fat, brain, and nerves, in one of the rabbits which lived a little over 4 hours, while in the other, which lived 18 hours, the subcutaneous tissues and fat alone were colored. It may be remarked that in several experiments with different dyes, the nervous system was free from dye at the end of 4 to 6 hours after the intravenous administration. In one experiment on a rabbit 0.125 gm. per kilo of benzeneazo-β-naphthylamine was administered intravenously in the form of an emulsion. The rabbit lived 3½ hours. On autopsy the dye was found in the fat, subcutaneous tissue, brain, nerves, medulla of the kidney, muscles, cartilage, and lungs. In similar experiments in which the duration of life was longer, the nervous system usually appeared normal in color. In a few cases, the nerves alone were stained. It might be added that the distribution of the dye was not restricted to fatty tissues and the nervous system. The femur was found markedly colored in one experiment after the intravenous injection of benzeneazoresorcinol; the bone marrow, however, was unstained in this case. The results concerning distribution may be briefly summarized by stating that the subcutaneous tissues and fat are generally colored after the administration of fat-soluble dyes. The brain may also contain dye occasionally, but the nerves are apt to be stained perhaps a little more frequently than the central nervous system.

We also made observations on the disappearance of dye from the blood. The tests were made with benzeneazoresorcinol only, which was given intravenously. In three experiments in which 25 and 67 mg. of the dye per kilo were introduced, it was still present in the serum after 9½ to 15 hours. This is especially interesting since, as it will be recalled, elimination of the dyes we examined ceased in the bile and in the urine in 4 to 5 hours after their administration. It seems quite probable, therefore, that the elimination of these bodies occurs only as long as there are excessive amounts in the circulation. It may also be pointed out in this connection that some of the tissues of the body were stained when the bile and the urine no longer showed the presence of dyestuff. This observation that fat-soluble dyes may be present in the blood and other tissues in visible form after they can no longer be seen in the excretions might be due to fatigue of the excretory mechanisms for these compounds which, as we have seen, seem to be exceedingly delicate and unstable. That a firm combination might be formed with lipoids in the tissues from which the dye is either separated with difficulty or is transformed into a leuko compound and thus escapes detection, also appears to be a plausible explanation.

VI. Experiments on Toxicity.

Although the present studies were mainly confined to inquiries on the fate of fat-soluble dyes in the body, observations were also made on the toxicity of these compounds. It might be stated in advance that none of the dyes examined were found to be very poisonous to the animals on which they had been tested. Thus, rabbits which received 1 to 2 gm. of benzeneazo-β-naphthylamine or of the toluene homologue (see Tables I and II) dissolved in oil and injected subcutaneously or intraperitoneally caused death in 4 to 9 days. A dose of 0.25 gm. per kilo was borne without manifestation of any untoward effects. It is interesting to observe that a dose of 1 gm. per kilo in oil given by mouth was more toxic than when given subcutaneously, the duration of life in one rabbit being 2¾ days, in another rabbit about 40 hours. As already mentioned, feeding experiments in rats were also carried out with these dyes. About 60 to 70 mg. per kilo of benzeneazo-β-naphthylamine fed daily with their food for 4½ months and about twice these amounts of tolueneazo-β-naphthylamine administered in the same way for 6½ months failed to show any poisonous action. Benzeneazoresorcinol (Table IV) given subcutaneously in amounts of 0.5 to 1.0 gm. per kilo likewise failed to induce any visible effect. A large number of experiments by intravenous injection were also made with this dye. 100 to 160 mg. per kilo dissolved in 1 per cent NaOH failed to produce any symptoms. When administered in the form of an emulsion, 100 mg. per kilo proved to be fatal to one rabbit in 4½ hours and to another in 18 hours. Two other rabbits which received 86 and 94 mg. per kilo survived. The administration of larger doses could not be carried out satisfactorily on account of the lack of a suitable solvent. Of the other members of the group (Table VI), benzeneazo-β-naphthol, is perhaps more toxic, as rabbits that received 0.64 to 0.55 gm. per kilo subcutaneously died in 2 to 18 days, loss of appetite being the only symptom noticed. That this dye was toxic was claimed also by Weyl (10), who fed it to a dog. The duration of life of rabbits that received 1.7 gm. per kilo of benzeneazobenzeneazo-β-naphthol (Table III) subcutaneously and intraperitoneally was 1 to 3 weeks, which makes it extremely doubtful whether death was due to the dye. Mendel and Daniels (9) state that large doses fed to cats were harmless provided the dye was pure. Benzeneazobenzeneazo-β-naphthol is therefore also much less active than benzeneazo-β-naphthylamine or the toluene compound. After 0.7 and 0.77 gm. of benzeneazophenol, loss of appetite was the only symptom observed. With the exception of one case (see asterisks, Table V) the rabbits survived. Its activity is therefore less than that of benzeneazo-β-naphthol. Benzeneazodimethylaniline dissolved in oil and given subcutaneously in doses of 0.53 to 0.6 gm. per kilo likewise failed to produce any symptoms except loss of appetite, which was observed in one rabbit. According to Weyl (10) this dye is not toxic to rabbits but Chlopin's (20) experiments on dogs which received 0.3 to 0.5 gm. per kilo by mouth, show that the dye may cause vomiting, diarrhea, and renal irritation. When such doses were repeated by him in 4 to 9 days he noticed severe symptoms of renal irritation and death.

Although the data on toxicity reported in the present investigation are of a preliminary character and are therefore inadequate, they nevertheless indicate important differences in the behavior of some of the dyes. As is shown in experiments with benzeneazobenzeneazo-β-naphthol, the toxicity of the naphthalene series may be decreased by the presence of another azo group, but even these compounds are more toxic than the phenol and resorcinol dyes. It may be remarked in this connection that Weyl (10), experimenting with a number of diazo colors, pronounced them non-poisonous.

SUMMARY.

1. Oil-soluble and water-insoluble dyes administered to different animals were eliminated in the urine and in the bile.
2. Elimination in the urine was usually inhibited in poisoning with zinc or oil of chenopodium.
3. Two of the compounds of benzeneazophenol and benzeneazoresorcinol, which were isolated from the urine of rabbits, proved to be conjugated with glucuronic acid.
4. Most of the dyes were deposited in the adipose tissues; staining of the nervous tissue, the kidney, and muscle was also observed in some experiments.
5. 10 to 15 hours after intravenous injection of 25 mg. per kilo of benzeneazoresorcinol, the dye was still present in the blood.
6. The toxicity of the different dyes was not pronounced even when larger doses were administered.

in the four cases noted.

In the tables S stands for subcutaneous, P for intraperitoneal, and V for intravenous. "Tissue" refers to subcutaneous tissue.

TABLE I.

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*		
1,175	♀	1.7	In oil.	P	42	4¾ days.	No autopsy.
1,615	♀	1.2	"	"	42	6¾ "	Bile fistula. Tissue and fat not stained. Liver normal. Kidney congested and brownish. No dye in bile after 6 days.
1,250	♀	3.2	Suspended in gelatin.	"	19	2⅔ "	Fat stained. Liver friable. Heart injected. Much fluid in peritoneal cavity. Bile duct highly stained.
1,015 (Cat.)	♀	2.0	Suspended in starch.	"		21 hrs.	Bile from bladder did not dye wool. Dye unabsorbed. Tissue, fat, brain, nerves, and muscle stained. Cat was fasted previously for 6 days. Loss of weight 22 per cent.
1,570 (Cat.)	♀	1.3	"	"	21	45 "	Cat was fed previously. Loss of appetite after injection. Tissue, fat, and nerves stained. Liver and kidney normal. Spinal cord and medulla white. Stomach contents not colored. Intestinal contents slightly colored. Brain not stained.
1,050	♂	2.0	In oil.	S	22	5 days.	Dye unabsorbed. Tissue slightly stained. Brain very slightly stained. Nerves and muscles not stained.

TABLE I—*Concluded.*

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*		
1,270	♀	0.25	In oil.	S	17	9 days after 2nd injection.	Duration of elimination, 17 days. Then 2nd injection, 1.0 gm. No autopsy.
1,165	♂	1.0	"	"	18	7¾ days.	Dye unabsorbed. Tissue not stained.
1,015	♂	1.0	Suspended in gelatin.	"	Faint, 17½	37 "	Elimination continued very faint till death. Animal chloroformed. Dye found encysted at site of injection. Tissue and muscle not stained. Kidney normal.
825	♀	1.0	In oil.	*Per os.*	17½	2¾ "	Loss of appetite. Mucosa of small intestine, stomach, tissue, and fat stained. Nerves and muscle not stained.
1,045	♀	0.125	Emulsified.	V	1½	3½ hrs.	Fed carrot diet, 250 gm. per day. Paresis in 1½ hrs. Tissue, fat, brain, nerves, and muscle stained. Kidney medulla stained. Cartilage, lungs, and peritoneal fluid also stained.
1,610	♀	0.062	"	"	1½		Very slight depression only symptom. Duration of elimination, 10 days. Animal discarded.

jection no symptoms were noted except loss of appetite in the four cases noted. The duration of elimination was until death except in the one case noted.

TABLE II.

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*	*days*	
1,675	♀	1.2	In oil.	P	20	2	Elimination in bile, dark red after 24 hrs. Bile fistula. Tissue and fat stained. Brain cortex and muscle slightly stained.
1,030	♂	2.0	"	"	19½	4	No autopsy.
1,200	♀	3.2	Suspended in gelatin.	"	18½	4	" "
1,850 (Cat.)	♂	1.1	Suspended in starch.	"	21	5¾	No appetite. Cat was fasted previously for 6 days. 13 per cent loss. Dye unabsorbed. Tissue slightly stained. Kidney normal. Peritoneal lining stained inside and out.
2,250 (Cat.)	♀	0.88	"	"	45 (21?)	9	Cat was fed previously. No appetite. Dye unabsorbed. Brain not stained. Tissue, fat, and nerves stained. Peritoneal lining stained inside and out.
1,070	♀	1.9	In oil.	S	22	5	Dye unabsorbed. Tissue, nerves, and muscle not stained.
1,160	♀	0.25	"	"	17	3¾ after 2nd injection.	Duration of elimination, 16 days. Then 2nd injection, 1.0 gm. Dye unabsorbed. Nerves not stained. Kidney slightly congested.
1,100	♂	1.8	Suspended in gelatin.	"	Faint, 17½	4	No appetite. Dye unabsorbed. Liver mottled.
950	♀	1.0	In oil.	*Per os.*	17½	1¾	No appetite. Tissue not stained. Stomach mucosa and secum slightly stained. Small intestine contained a little unabsorbed dye.

Experiments with Benzeneazobenzenezao-β-naphthol (*Table III*).—Four rabbits were used. After the injection no symptoms were observed except loss of appetite in the first two cases. The duration of elimination was until death.

TABLE III.

Animal.		Dye administered.			Elimination after:	Duration of life.	Remarks.
Weight.	Sex.	Per kg.	Mixture.	Injection.			
gm.		*gm.*			*hrs.*	*days*	
1,200	♀	1.7	Suspended in gelatin.	P	17	7¾	Some dye unabsorbed. Tissue and brain not stained. Kidney congested.
1,240	♀	1.7	"	S	Faint, 17	20	Some dye unabsorbed. Tissue slightly stained.
1,215	♂	1.7	In oil.	"	Faint, 22	14	Bile dark wine-colored. No dye in bile, large and small intestine, or stomach. Brain not stained. Kidney congested.
1,900	♀	0.026	Emulsified.	V	70 min.*	22 hrs.	Bile fistula. Elimination in bile began in 14 min. Colored at death. Very slow secretion of bile, 4 cc. in 5¼ hrs. Before injection, secreted 8 cc. in 1 hr.

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* No urine obtainable sooner.

Experiments with Benzeneazoresorcinol (Table IV).—The data for nineteen rabbits are shown in the table.* The injection was intravenous in the first twelve cases, subcutaneous in the rest.

TABLE IV.

Animal.		Dye administered.		Elimination after:	Duration of:		Remarks.
Weight.	Sex.	Per kg.	Mixture.		Elimination.	Life.	
gm.		*gm.*		*min.*	*hrs.*		
1,610	♀	0.094	Emulsified.	10	23	72 hrs.	Injection in divided doses. 31 mg. per kg. injected at a dose. Animal chloroformed. Tissue and fat stained. Fatty degeneration of lumbar muscles. Nerves not stained.
1,735	♀	0.029	"	15	4½	6 "	Animal chloroformed. Tissue and fat stained. Urine in bladder normal. Nerves not stained.
		0.029	"	10	4–5		
1,530	♀	0.100	In NaOH.	9	±48		Discarded. Autopsy 9 days after injection. Tissue and fat slightly stained. Bile and urine normal.
1,700	♀	0.100	"	9	±24	72 "	Two injections in 15 min. Animal chloroformed. Tissue and fat stained. Femur stained inside and out. Bone marrow not stained.
1,910	♀	0.026	"		5–6		Discarded.
1,890	♀	0.026	"	15	3–4		"
1,730	♂	0.028	"	15	±5	4 days.	Animal chloroformed. Tissue and fat slightly stained. Blood in urine obscured accurate observation.
1,470	♂	0.013	"	25	3		Fasted 7 days before injection. Discarded.
1,790	♀	0.067	"			22 hrs.	Tissue stained. Serum stained 10 hrs. after injection.
1,970	♀	0.025	"			22 "	Bile light red-brown. Tissue and fat stained. Urine in bladder normal. Serum stained 9½ hrs. after injection.

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* The total number of rabbits employed in experiments with benzeneazoresorcinol was thirty-four.

TABLE IV—*Concluded.*

Animal.		Dye administered.		Elimination after:	Duration of:		Remarks.
Weight.	Sex.	Per kg.	Mixture		Elimination.	Life.	
gm.		*gm.*		*min.*	*hrs.*		
1,985	♂	0.025	In NaOH.			20 hrs.	Animal chloroformed. Tissue stained. Fat slightly stained. Urine in bladder normal. Serum stained 15 hrs. after injection, though bile was normal in color.
2,330	♀	0.086	Emulsified.	10	±72	4 days.	Animal chloroformed. Kidney normal. Urine normal. Spleen very hard, brittle, and *yellow*. Tissue slightly stained. Fat, brain, and nerves not stained.
				hrs.	*days*		
2,030	♀	0.5	In oil.	3			Brain and nerves not stained. Dye in urine disappeared in 2 days. Treated with chenopodium. Dye unabsorbed.
1,300	♂	0.75	"	2			Treated with chenopodium. Dye unabsorbed.
1,950	♀	0.5	"	17	60		Survived and was discarded.
1,075	♀	1.0	"	17	60		" " " "
2,910	♀	0.28	"	½	8	8 "	Animal chloroformed. Fat slightly stained. Adrenals seemed stained, chyle cream-colored. Urine normal. Dye unabsorbed.
1,980	♂	0.2	In NaOH.	17	12	13 "	Bile fistula.
2,150	♀	0.23	"	1			Given hydrazine.

Experiments with Benzeneazophenol (*Table V*).—The data for four rabbits are shown in the table.* No symptoms were observed except loss of appetite in the first case.

TABLE V.

Animal.		Dye administered.		Elimination after:	Remarks.
Weight.	Sex.	Per kg.	Mixture.		
gm.		*gm.*		*hrs.*	
1,400	♀	0.7	In oil.	5	Oil of chenopodium given the day after dye. Elimination of dye inhibited in 72 hrs. Rabbit given 1.0 gm. of dye 4 weeks later. Lived 3 days.
1,960	♂	0.5	"	4	Oil of chenopodium given 4 days after dye. Elimination of dye inhibited in 24 hrs. Survived.
1,910	♀	0.7	"	3	Lived 5 days. Urine in bladder contained dye; no dye in bile.**
1,165	♀	0.43	In NaOH.	17½	Bile in gall bladder normal color.

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* The total number of rabbits employed in experiments with benzeneazophenol was seven.

** See page 418.

Experiments with Benzeneazo-β-naphthol (*Table VI*).—Ten rabbits were used, all males except the first. The injection was subcutaneous in all but the last three cases, in which it was intravenous. Loss of appetite was the only symptom observed. The duration of elimination was until death unless otherwise noted.

TABLE VI.

Weight of animal.	Dye per kg.	Elimination after:	Duration of life.	Remarks.
gm.	*gm.*	*hrs.*		
1,325	0.75	21	9 days.	No elimination in urine after 4½ hrs. Oil of chenopodium on 6th day; elimination of dye markedly decreased. Some dye not absorbed. Tissue stained. Nerves and muscles not stained.
1,680	0.59	17	11 "	
1,570	0.64	1	48 hrs.	Dye present in bile, liver, and kidney. Fat stained. Absorption incomplete. Tissue not stained.
1,800	0.55	17	18 days.	Dye present in bile.
1,995	0.5	17	13 "	Bile fistula made 13 days after injection. Dye present in bile.
1,670	0.6	21	7 "	Fasted 5 days before injection. Some dye unabsorbed. Tissue and fat stained.
1,855	0.55	21	12 "	Fasted 5 days before injection. Some dye unabsorbed. Tissue and fat stained. Brain and nerves not stained. Dye present in bile.
1,740	0.029	¾	4½ hrs.	Bile fistula. 16 min. after injection, dye in bile but secretion of bile stopped.
2,270	0.022		19 "	Bile fistula. Dye present 16 min. after injection. Secretion of bile slow. Urine secreted over night contained small amount of dye.
1,150	0.043	1	21½ "	Bile fistula. Dye appeared 8 min. after injection. Bile secretion before injection, 5 cc. in 35 min.; after injection, 4 cc. in 5 hrs., and none after this.

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AN ELECTRICALLY HEATED VACUUM DESICCATOR.*

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In preparing certain biochemical products it is frequently desirable to employ a desiccator of large capacity, capable of evacuation and of being heated and maintained at a predetermined temperature. The various forms of apparatus available on the market are either of very small capacity or else expensive and deficient in the range and adjustability of the temperatures employed. We accordingly have had constructed for our use the apparatus described below, which is of large capacity, readily accommodating a filter funnel 9 inches in diameter containing a 50 cm. filter paper. It maintains a vacuum for several hours and may be held with considerable precision at any desired temperature within a wide range. If desired, a vessel containing sulfuric acid or other desiccating reagent may be placed on the floor of the apparatus and false bottoms or shelves may be fixed at any desired height above the reagent. With the aid of this apparatus, employing certain obvious precautions, tethelin suspended in alcohol-ether mixture may be completely dried without discoloration at 36°C. within 6 hours, yet this substance is so exceedingly hygroscopic that a few minutes' exposure to the air of the room suffices to convert it into a deeply colored, sticky mass.[1]

The apparatus consists of a double walled circular copper ($\frac{1}{16}$ inch sheet copper) chamber, well insulated by asbestos and set in a monel metal shell. This chamber can be closed at the top

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* Aided in part by a grant from the George Williams Hooper Foundation for Medical Research.

[1] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 409.

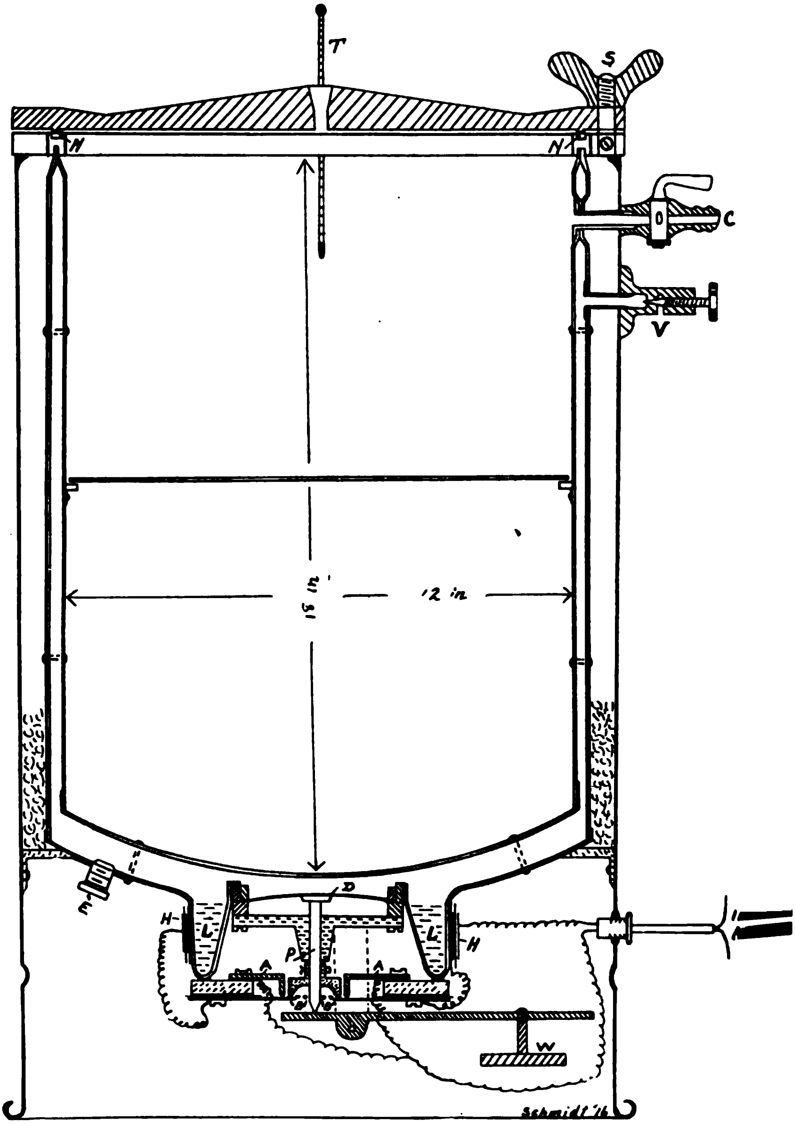

FIG. 1. An electrically heated vacuum desiccator. Scale $\frac{1}{5}$.

by a tight fitting cast aluminum lid which can be screwed down by means of three screws (S). A rubber gasket in the notched space (N) makes the compartment air-tight. The chamber is evacuated by attaching a vacuum pump to the cock (C) and the vacuum so obtained can readily be maintained for some time after the cock is closed. The desired temperature in the evacuated chamber is maintained by the heated vapor of dichloromethane (boiling point 40°C.) which fills the space between the two copper walls, heat being furnished by eight 25 watt nichrome heating elements, connected in parallel and placed around the pocket (L). Each heating element is in turn connected to a small platinum-tipped brass spring (B) which makes contact with a platinum point set on the ring (A). In this way one or more of the heating elements can be used as needed.

Temperature control is maintained by means of the sprung copper disc (D). When heat is applied to the liquid dichloromethane in (L), vapor is formed which will exert a pressure on the copper disc (D), tending to force it downward. This disc n turn presses against the pin (P) which when forced downward will cause the contact between (A) and (B) to be broken. In this way any or all of the heating elements (H) are switched n or off as needed to maintain a constant vapor pressure and temperature in the double walled space surrounding the chamber. By shifting the weight (W), tension on the disc (D) may be increased or decreased and the temperature in the chamber varied (within a certain range) at will. By selecting liquids having a boiling point higher than that of dichloromethane the emperature range may also be varied. Heating of the chamber by means of the vapor of a volatile liquid assures a uniform distribution of heat.

On using the apparatus for the first time the needle valve (V) s opened to allow air to escape and closed again when dichloromethane vapor begins to escape. This assures the space being lled solely with dichloromethane vapor. To fill or drain the quid in (L) a drain plug (E) is provided. To insure rigidity and ightness, the walls are thoroughly braced and reinforced and ll joints are silver-soldered.

The apparatus was constructed by Mr. A. J. Kercher of Berkeley and embodies several of his own inventions.

THE OXIDATION OF BRANCHED CHAIN FATTY ACIDS.

I. THE ACTION OF HYDROGEN PEROXIDE ON THE HOMOLOGUES OF ISOBUTYRIC ACID.

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(Received for publication, September 25, 1916.)

It is noteworthy that while the majority of fatty acids occuring in nature belong to the normal series, those composed of more han twenty-two carbon atoms contain at least one tertiary arbon atom in their molecule.[1] The structure of these acids is ntirely unknown. The problem of their structure naturally s identical with that of the location of the tertiary carbon atom n the molecule. There exist two methods by means of which he solution of the latter problem may be reached. One conists in the gradual transformation of a fatty acid into its ower homologue passing through the following phases:

$$RCH_2COOH \rightarrow RCHBrCOOH \rightarrow RCHOHCOOH \rightarrow RCOOH^2$$

By continuing these operations on the lower homologues one may inally reach an acid of known structure, or one the structure of which can be made known by synthesis. This method has the advantage of generality and reliability; however, it has the disadvantage of requiring large quantities of material and much time or execution.

The second possible method is to be based on the properties of the tertiary carbon atom. In aliphatic hydrocarbons, alcohols, acids, etc., containing a tertiary carbon atom (≡ CH), this is ound to be most susceptible to the action of oxidizing agents.

However, the experience gained in this laboratory on lignoceric and cerebronic acids was rather disappointing. Lignoceric acid was shown by Meyer, Brod, and Soyka[3] to contain a tertiary carbon atom. Levene and West[4] have substantiated this conclusion and have also demonstrated the presence of a tertiary carbon atom in cerebronic acid. Yet when lignoceric or cerebronic acid was heated for a considerable period of time in a permanganate solution one could find no evidence of the oxidation of the tertiary carbon atom.

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[1] See Meyer, V., and Jacobson, P., Lehrb. organ. Chem., Leipsic, 2nd edition, 1907, i, 501.

[2] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1914, xvi, 475.

On the other hand, there is no reason to doubt that under favorable conditions this oxidation may take place. The problem then, as it presented itself at this phase, was to establish conditions under which the tertiary carbon atom undergoes oxidation readily. The undertaking seemed all the more desirable since the conclusions regarding the properties of the methenyl group ($\equiv$ CH) of the earlier writers were based on a very limited number of observations. It was realized that the character of the radicles attached to the tertiary carbon atom may be of considerable influence on its stability. In the series of aliphatic acids the distance of the tertiary carbon atom from the carboxyl seemed particularly worthy of consideration. In view of this it was concluded to investigate the action of oxidizing agents on the higher homologues of dimethylacetic (isobutyric) acid $(CH_3)_2CH \cdot COOH$. Unfortunately only the lower members of this series had been prepared by previous workers and our task fell into two parts, one the preparation of the higher homologues and the other their oxidation.

Preparation of the Acids.

The preparation of the acids was based on the following se of reactions:

$$RCH_2OH \rightarrow RCH_2I \rightarrow RCH_2CH \cdot (COOC_2H_5)_2 \rightarrow RCH(COOH)_2 \rightarrow$$
$$RCH_2COOH \rightarrow RCH_2COOC_2H_5 \rightarrow RCH_2CH_2OH$$

The set of reactions was then continued progressively to build up the higher homologues.

2-Methylpropyl alcohol (isobutyl alcohol) $(CH_3)_2CH \cdot CH_2OH$

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[3] Meyer, H., Brod, L., and Soyka, W., *Monatsh. Chem.*, 1913, xxxi 1113.

[4] Levene and West, *J. Biol. Chem.*, 1914, xviii, 477.

and its next higher homologue, 3-methylbutyl alcohol (isoamyl alcohol) $(CH_3)_2CHCH_2 . CH_2OH$, served as the starting material.

The first substance was obtained from Kahlbaum and from the Du Pont Chemical Works, and purified by fractional distillation. The second was prepared from the so called "amyl alcohol," following the procedure first suggested by Pasteur and later perfected by Marckwald.[5] The directions of the latter investigation were followed rather in principle than in detail. After some experience in handling the large volumes of liquids the yield was very satisfactory.

The iodides were prepared by distilling the alcohols with hydriodic acid. The procedure was essentially the same as recommended by Norris.[6] Only such slight modifications were introduced as suggested themselves by the larger quantities of material employed in course of this work. The details are given in the experimental part. The yields were never less satisfactory than those obtained by Norris, and were at times considerably better.

Reduction of the Esters to Alcohols.—For the reduction of esters into the corresponding alcohols the process of Bouveault and Blanc[7] has been essentially improved. In the original process an alcoholic solution of the ester is slowly added from a dropping funnel to six atoms of sodium divided in large pieces. The procedure was rather awkward and the efficiency of the sodium minimal. In the present work the sodium was suspended in toluene. The flask was provided with a mechanical stirrer. By heating the mixture in a metal bath with continuous stirring the sodium was reduced to small globules. When this stage was reached, alcohol and then the ester in alcoholic solution were allowed to flow from a dropping funnel into the emulsion of sodium and toluene. By continuous stirring the contact surface between sodium and ester was maintained at its maximum. The time of reaction is reduced from 6 hours by the original method to 1 hour by the modified method. The apparatus and the details of the procedure are described in the experimental part.

All the other steps in the preparation of the acids were those of the malonic ester synthesis, and were carried out in the conventional way.

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[5] Marckwald, W., *Ber. chem. Ges.*, 1901, xxxiv, 479; 1902, xxxv, 1595.

[6] Norris, J. F., *Am. Chem. J.*, 1907, xxxviii, 627. Norris, J. F., Watt. M., and Thomas, R., *J. Am. Chem. Soc.*, 1916, xxxviii, 1071.

[7] Bouveault, L., and Blanc, G., *Bull. Soc. chim.*, 1904, xxxi, 666.

In the course of this work the acids, esters, alcohols, iodides, and substituted malonic acids of eight homologues of isopropane were prepared. The isobutyl and isoamyl derivatives had for their starting materials the corresponding alcohols. The normal changes in physical properties with an increase in the number of carbon atoms in the chain were noted. The specific gravities of the alcohols increased progressively. The solubility in water of the dibasic acids decreased and their solubility in petroleum ether increased with an increase in the hydrocarbon part of the molecule. The melting points of the dibasic acids, the phenylurethanes of the alcohols, and the amides of the monobasic acids showed the customary irregularity. Table I shows the record of the constants of the new acids and of the intermediate products in the preparation of the acids.

TABLE I.

Physical Constants of Compounds with the Radicle $(CH_3)_2CH(CH_2)x$.*

No. of carbons in R.†	RCH_2OH b. p.	$C_6H_5NH.CO.OCH_2.R$ m. p.	RCH_2I b. p.	RCOOH b. p.	$RCOOC_2H_5$ b. p.	$RCONH_2$ m. p.	$RCH(COOC_2H_5)_2$ b. p.	$RCH(COOH)_2$ m. p.
4	130‡	56.6	148‡	174‡	134.3‡	127–129‡	240–242‡	93
5	153	48.0	173.2	199.3‡	160.4‡	120‡	137.0_{11}	86.5
6	170.5	82.5	195.2	216	182.7	103.5–104.0	155_{15}	100.0–100.3
7	188.5	81.0–81.4	100.0_{17}	$126–127_{14}$ 232	200.3	114.0	171.8_{18}	89.5–90.0
8	206.0	66.4	120.0_{20}	140.5_{15} 248	220.5	106.5	182_{18}	92
9				155.6_{16}				
10				$174\ 0–174.5_{23}$				

* All data taken with standard thermometer and corrected.

† R = $(CH_3)_2CH(CH_2)x$.

‡ Taken from the literature and in every case where the compound was prepared, verified by this work.

Oxidations.

In selecting the oxidizing agent there was a choice of nitric acid, chromic acid, permanganate, or hydrogen peroxide. Every agent has advantages and disadvantages. On oxidation with nitric acid the reaction is complicated by the formation of nitro derivatives.[8] The oxidation with potassium permanganate may, according to Przewalsky,[9] give rise to isomerization. Chromic acid also has some disadvantages, particularly because the reagent adheres tenaciously to the products of oxidation. However, it is planned to test systematically the individual peculiarities of each of these agents. The present work was limited to experiments with hydrogen peroxide. It was selected because it presents the greatest convenience in manipulation.

From the work of Dakin[10] it is known that normal fatty acids undergo the so called β oxidation, the acid giving rise to a ketone having in its carbon chain one atom of carbon less than the original acid:

$$RCH_2CH_2COOH \rightarrow RCOCH_3$$

To a smaller degree they oxidize in the α-position with the formation of the next lower aldehyde:

$$RCH_2COOH \rightarrow RCHO$$

Regarding the action of hydrogen peroxide on the branched chain fatty acids the observations are limited to those of Dakin on isobutyric and isovaleric, and to those of Raper on isobutyric and on α-methylbutyric acids. In isovaleric acid the tertiary carbon atom happens to be situated in the β-position, which renders it doubly susceptible to the action of hydrogen peroxide. Raper,[11] reports besides the usual oxidation of the tertiary carbon atom, also a peculiar behavior of the α-methyl group. The present work does not deal with acids of this structure and hence this new type of oxidation will be omitted from the present discussion. However, the fact that the predominating product of oxidation of α-methylbutyric acid was methylethyl ketone goes to show that in this substance the tertiary carbon atom is the principal locus of oxidation.

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[8] Bredt, J., *Ber. chem. Ges.*, 1882, xv, 2324.

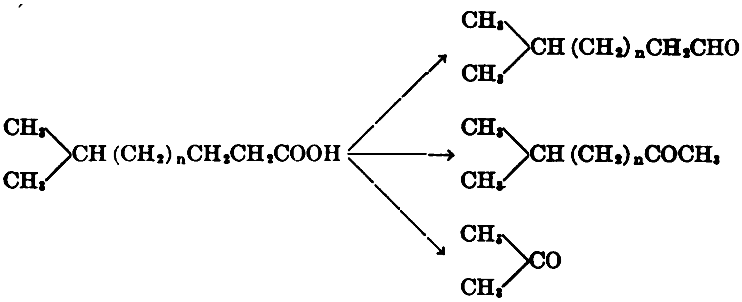
[9] Przewalsky, E., *J. prakt. Chem.*, 1913, lxxxviii, 495.

[10] Dakin, H. D., Oxidations and Reductions in the Animal Body, London, 1912.

[11] Raper, H. S., *Biochem. J.*, 1914, viii, 320.

In course of the present investigation the higher homologues of isobutyric acid (including 9-methyldecylic acid) were subjected to the action of hydrogen peroxide.

On the basis of facts which have been referred to in this discussion, the following products of oxidation were to be expected.



The principal aim of the work was to find the conditions which favor the third reaction above the other two, and furthermore to investigate the influence on this reaction of the distance of the tertiary carbon atom from the carboxyl group.

It was found that the reaction proceeded most favorably when the solution of the organic acid was slightly acid and when an amount of peroxide solution equivalent to three and one-half atoms of oxygen was employed for the reaction. Under such conditions all of the three reactions outlined above took place. However, acetone was the predominating product of reaction. Acetone was identified as its *p*-nitrophenylhydrazone, which showed the correct melting point and composition.

The presence of the higher ketones was demonstrated by an indirect method; the value of the mixed ketones was determined and from this was deducted the value of the acetone present in the same solution.

The aldehydes resulting from the oxidation in the α-position were identified as the corresponding acids.

The plan of the analytical work was essentially the same as employed by Dakin and by Raper, and is given in detail in the experimental part.

As regards the yields of acetone, there was noted a progressive fall with the increase of the distance between the tertiary carbon atom and the carboxyl group. A marked exception to the rule was observed in the case of isobutyric and isovaleric acids. Contrary to the general rule the yield of acetone was greater from the higher homologue. Undoubtedly this is occasioned by two factors. First, in isovaleric acid the acetone is formed by two types of oxidation; the β oxidation, and the oxidation of the tertiary carbon atom as such; on the other hand, in isobutyric acid the β oxidation leads to demethylation and hence proportionally diminshes the mass of the tertiary carbon atoms. The demethylation of isobutyric acid was demonstrated by Raper, who found propionic aldehyde among the products of oxidation of this acid. In the present work propionic acid was found directly among the products of oxidation.

Incidentally it may be mentioned that all attempts to isolate dicarboxylic acids from the non-volatile part of the oxidation product were futile. Furthermore, the acids extracted from this fraction on titration gave molecular weight values correspondng closely to the original acids. Hence it seems possible that the remainder of the molecule after the formation of acetone undergoes complete oxidation to carbon dioxide.

EXPERIMENTAL PART.

3-Methylbutyl Alcohol (*Isoamyl Alcohol*).—Constant boiling amyl alcohol having a rotation of −0.75°, indicating 12.7 per cent of the active alcohol, was mixed with an equal weight of concentrated sulfuric acid. The temperature during the mixing was kept below 40°C. The next day water and ice were added until the liquid had been diluted to double its volume. Solid barium hydroxide was then added with mechanical stirring until the liquid was neutral to Congo red. During this process also, the temperature was kept below 40°C. The precipitated barium sulfate was washed by decantation until all of the barium amyl sulfate had been removed. The wash liquors were repeatedly concentrated under diminished pressure and allowed to crystallize until the resulting crystals showed a greater rotation than +0.20° in 10 per cent solution in a 2 dm. tube at room temperature. All mother liquors and crystals having a rotation greater than this were rejected. As barium amyl sulfate of this purity makes about a 10 per cent solution in water at room temperature the rotation of the mother liquors and of the crystals under the conditions stated above are comparable.

About 25 per cent of the alcohol was recovered from the distillates of the wash liquors. The weight of the crude salt was approximately equal to the weight of the alcohol originally taken. The first yield of crystals from the wash liquors had a rotation of 0.05 to 0.08°. The barium amyl sulfate was recrystallized either from two and a half times its weight of water or from mother liquors of higher purity until it showed no rotation in 10 per cent solution in a 2 dm. tube. After this it was recrystallized once from water. All the mother liquors not used for recrystallizing were concentrated to crystallization.

The inactive salt was decomposed in hot concentrated solution with a moderate excess of strong sulfuric acid. The precipitated barium sulfate was washed by decantation and the wash liquors refluxed for 3 hours to hydrolyze the isoamyl sulfate. A layer of alcohol separated out. This was removed. The alcohol dissolved in the aqueous layer was recovered by distillation. The alcohol obtained in this manner contained as impurities, sulfuric acid and isoamyl sulfate. For purification two-thirds of the original volume were distilled off directly and the remainder prior to distillation was freed from impurities by washing with water.

The alcohol obtained in this way had a rotation of 0.04 to 0.08° in a 1 dm. tube at room temperature. The yield was from 10 to 15 per cent. 4 kilos of inactive alcohol were used in the course of the following work.

3-Methylbutyl Phenylurethane.—2 gm. of inactive alcohol and 2.7 gm. of phenylisocyanate were heated under a reflux for a few minutes. The liquid solidified on cooling. After four crystallizations from dilute alcohol the melting point showed no further change. Melting point 56.6°C. (corrected).

0.2006 gm. substance neutralized 9.57 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{12}H_{17}NO_2$:	Found:
N....................................	6.76	6.63

3-Methylbutyl Iodide.—50 gm. lots of the inactive alcohol were slowly distilled with 224 cc. (three equivalents) of constant boiling hydriodic acid. The distillation was continued until the temperature reached 127°C. At this point all of the iodide had distilled over. A new portion of the alcohol and enough acid to make up the original volume were then added and the distillation was continued. The iodide was separated from the distillate, washed with water, decolorized with sodium thiosulfate, dried over sodium sulfate, and distilled. The low boiling portions were redistilled with hydriodic acid. The yield was 88 per cent of the theory. 750 gm. of the alcohol gave 1,485 gm. of the iodide.

5-Methylhexylic Acid.—3-Methylbutyl iodide was condensed in the customary manner with malonic ester in lots of 50 to 100 gm. The 3-methylbutylmalonic ester was washed with water and then saponified without further purification.

For saponification the wet ester was vigorously stirred with twice the theoretical amount of 50 per cent sodium hydroxide. The mass soon became hot and then solidified. The evaporation of the alcohol and water kept the temperature below 100° and additional cooling was not required even when lots of 800 gm. were saponified. The soap was extracted twice with acetone, then mixed with a small amount of water and acidified with concentrated hydrochloric acid with cooling. A large part of the 3-methylbutylmalonic acid separated out and later solidified. The sodium chloride was filtered off and extracted twice with ether, the mother liquors were extracted twice with ether in a separatory funnel and then for 4 hours in Thiele continuous extractors. From the latter extract about 10 per cent of the yield was obtained. For later preparations the acid dissolved in the mother liquors was recovered more conveniently but less economically by precipitating it from ammoniacal solution as the calcium salt. The ether solution of the acid was dried, the ether removed, and the syrup crystallized over sulfuric acid in a vacuum desiccator. The crystals were washed with petroleum ether. The constants found for this acid and its ethyl ester agreed with those reported by the earlier workers.[12] The acid melts without decomposition at 93°C. The yield was 83 per cent of the theory, calculated on the basis of the iodide. 1,300 gm. of 3-methylbutyl iodide gave 1,000 gm. of the dibasic acid. The acid crystallizes readily from benzene. It dissolves readily in water and is almost insoluble in petroleum ether.

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[12] Paal, C., and Hoffmann, T., *Ber. chem. Ges.*, 1890, xxiii, 1496. Frankland, E., and Duppa, P. F., *Ann. Chem.*, 1866, cxxxviii, 339. Fournier, H., *Bull. Soc. chim.*, 1909, i, 925. Nef, J. U., *Ann. Chem.*, 1901, cccxviii, 146. Grimshaw, H., *Ann. Chem.*, 1873, clxvi, 168. Wallach, O., *Ann. Chem.*, 1915, cdviii, 190.

The dibasic acid was decomposed in 100 gm. lots by heating in a distilling flask at 170–180° for half an hour. The temperature was then raised and the 5-methylhexylic acid distilled. 410 gm. of crude dibasic acid gave 263 gm. of the acid. The yield of 5-methylhexylic acid on the basis of the fraction boiling at 214–216° (corrected) was 85 per cent of the theory. The 5-methylhexylic acid was neutralized with sodium hydroxide and the solution distilled with steam to free the soap from impurities. 5-Methylhexylic acid has been prepared by several workers.[12] There is a lack of agreement between the boiling points reported by them, the variation ranging between 208 and 214–216°C. The boiling point of the substance described in this report was 216°C (corrected) at 762 mm. pressure.

0.1327 gm. substance gave 0.3130 gm. CO_2 and 0.1301 gm. H_2O.

	Calculated for $C_7H_{14}O_2$:	Found:
C	64.57	64.32
H	10.85	10.98

Ethyl 5-Methylhexylate.—The acid was esterified by refluxing for 3 hours with three equivalents of absolute alcohol containing 5 per cent of sulfuric acid. The high boiling fraction was re-esterified. The yield was 95 per cent of the theory. The boiling point given by Paal and Hoffmann[12] is 177°C. (uncorrected). Their analytical data are not entirely satisfactory. The boiling point found on distilling several large lots was 182.7°C. (corrected) at 750 mm. pressure.

0.0978 gm. substance gave 0.2440 gm. CO_2 and 0.0998 gm. H_2O.

	Calculated for $C_9H_{18}O_2$:	Found:
C	68.29	68.05
H	11.48	11.43

5-Methylhexylic Amide.—The acid chloride was prepared by heating 8 gm. of 5-methylhexylic acid with 7.3 gm. of thionyl chloride. The acid chloride was then poured into ice-cold ammonium hydroxide. The amide was filtered off, dissolved in ether, and the solution filtered. The ether was removed and the amide crystallized. Benzene was found an unsatisfactory solvent as eight crystallizations from this solvent were necessary for purification. Melting point 103.5–104.0°C. (corrected).

0.1013 gm. substance gave 0.2414 gm. CO_2 and 0.1074 gm. H_2O.

	Calculated for $C_7H_{15}NO$:	Found:
C	65.04	64.99
H	11.71	11.87

5-Methylhexyl Alcohol.—The alcohol was prepared by reducing 30 gm. lots of 5-methylhexylic ester with sodium and alcohol in the following manner. The reductions are carried out in a 500 cc. wide-necked, round-bottomed flask fitted with a mechanical stirrer as shown in Fig. 1. The upper half of the mercury seal revolves and is the only part of the apparatus to which the stirring rod is attached. 50 cc. of toluene, dried over sodium, are placed in the flask with six equivalents of metallic sodium. The flask is heated until the toluene refluxes. The stirrer is then rotated vigorously until the melted sodium is divided into a fine emulsion with the toluene. Then while the stirrer is still running, the metal bath is removed and a few cc. of absolute alcohol are allowed to run into the flask through the dropping funnel. Following this the ester, dissolved in three equivalents of alcohol, is rapidly introduced through the same funnel. The best results are obtained if all of this solution is added within 10 or 15 minutes. It is at first necessary to cool the reaction flask with a bath of cold water which is removed as soon as the reaction moderates to such a degree that the reflux condenser is capable of condensing the alcohol vapor. When refluxing has nearly ceased—after 20 to 30 minutes—more absolute alcohol is slowly added. When this no longer causes refluxing the flask is again heated with a metal bath. This is continued with the occasional addition of alcohol until the sodium is completely dissolved. At this time the contents of the flask are entirely in solution. The reaction should be completed in about an hour. If it proceeds more rapidly than this some ester may escape reduction or saponification and may appear as an impurity of the higher alcohol. It is always essential to have a considerable degree of

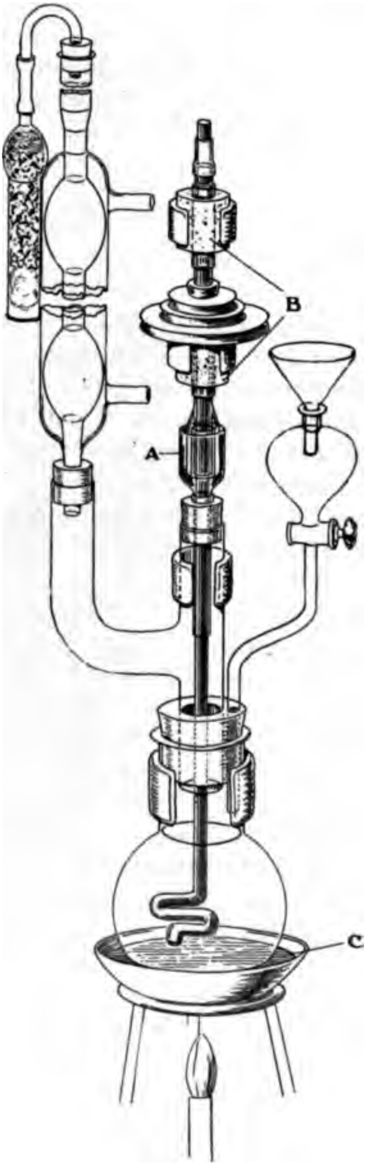

Fig. 1. Apparatus for the reduction of esters to alcohols.

ution as otherwise the yield of the new alcohol is impaired rough side reactions. The quantity of absolute alcohol used the reduction is generally about 250 cc. The toluene aids e reaction not only by permitting the melting and emulsification the sodium but also by dissolving the soap formed in course the reaction, thus keeping the entire mass liquid.

After the reduction is completed the liquid is cooled, diluted th cold water, and rapidly distilled with steam. The first porns of the distillate contain practically pure ethyl alcohol and luene. When the greater portion of these has been distilled over d the upper layer of liquid in the distilling flask has been reduced a small volume the further distillate acquires the odor of the w alcohol. The receiver is then changed and soon the dislate turns cloudy and contains water and the new alcohol with tle ethyl alcohol and with little or no toluene. Occasionally ter all of the new alcohol has been distilled over, a small amount dark colored material remains floating in the distilling flask. is consists of condensation products and other reduction oducts from the ester and does not appear if the reduction has en properly carried out. It is not necessary to remove it fore recovering the acid resulting from saponification of the ter. This is done by acidifying and subsequently distilling the id out with steam.

The alcohol-toluene distillate was fractionated through a five lb 18 inch column. All but a small portion distilled at 78–79°. e residue left in the distilling flask after the temperature has ached 80° is added to the distillate containing the new alcohol. om 5 to 10 per cent of the yield is obtained in this way.

With alcohols of six to nine carbons the yield is 60 to 65 per nt of the theory, calculated on the basis of the ester used. If e recovered acid is taken into consideration the yield is 90 95 per cent. Lauric ester gave a slightly smaller yield.

5-Methylhexyl alcohol is very slightly soluble in water and in nstant boiling hydriodic acid. Boiling point 170.5°C. (corrected) 755 mm. pressure. Specific gravity at 25°, 0.8192. A 1.6 cc. knometer was used.

0.1018 gm. substance gave 0.2710 gm. CO_2 and 0.1248 gm. H_2O.

	Calculated for $C_7H_{16}O$:	Found:
C	72.33	72.53
H	13.88	13.74

5-Methylhexyl Phenylurethane.—The urethane was prepared from 3 gm. of alcohol in the manner described under 3-methylbutyl phenylurethane. The melting point was constant after three crystallizations from dilute alcohol. Melting point 82.5°C. (corrected).

0.1993 gm. substance neutralized 8.41 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{14}H_{21}NO_2$:	Found:
N	5.95	5.91

5-Methylhexyl Iodide.—75 gm. lots of 5-methylhexyl alcohol were converted into the iodide in the manner described under 3-methylbutyl iodide. The yield was 85 per cent of the theory. The iodide is to a large degree soluble in absolute methyl alcohol. Boiling point 195.0–195.2°C. (corrected) at 765 mm. pressure.

0.1921 gm. substance gave 0.1998 gm. AgI (Carius).

	Calculated for $C_7H_{15}I$:	Found:
I	56.15	56.20

Ethyl 5-Methylhexylmalonate.—5-Methylhexyl iodide was condensed with malonic ester in 50 gm. lots. A portion of the ester was washed with water, dried, and distilled three times under diminished pressure. Boiling point 155°C. (corrected) at 15 mm. pressure.

0.1237 gm. substance gave 0.2964 gm. CO_2 and 0.1134 gm. H_2O.

	Calculated for $C_{14}H_{26}O_4$:	Found:
C	65.06	65.25
H	10.15	10.26

5-Methylhexylmalonic Acid.—The crude ester was saponified and the acid recovered from the soap in the manner described. The lesser solubility in water of 5-methylhexylmalonic acid and of its calcium salt made the use of the continuous extractors unnecessary. The acid is somewhat more soluble in petroleum ether than is 3-methylbutylmalonic acid, but this solvent was still found satisfactory for washing the crystals. The substance crystallizes readily from benzene. Melting point 100.0–100.3°C. (corrected) without decomposition.

0.1380 gm. substance gave 0.2997 gm. CO_2 and 0.1131 gm. H_2O.
0.6218 " " neutralized 61.58 cc. 0.1 N NaOH.

	Calculated for $C_{10}H_{18}O_4$:	Found:
C	59.35	59.20
H	8.96	9.17
Mol. Wt	202.1	201.9

0.5 gm. of the acid was shaken with 50 cc. of water for 4 hours on 2 consecutive days and then allowed to settle over night. A portion of the clear solution was removed, passed rapidly through a filter and titrated. 10 cc. of solution neutralized 4.11 cc. 0.1 N NaOH, which is equivalent to 0.0415 gm. of the acid. The solubility at 24.5° is therefore 4.15 gm. per liter.

7-Methyloctylic Acid.—The 5-methylhexylmalonic acid was decomposed in the usual manner. After the first distillation the 7-methyloctylic acid was neutralized with sodium hydroxide and the solution extractedt wice with ether to remove impurities. The acid was then recovered from the salt and distilled twice under diminished pressure. It is very slightly soluble in water and is somewhat volatile with steam. Boiling point 248°C. (corrected) at 765 mm. pressure and 140.5°C. at 15 mm. pressure. The acid solidifies in the refrigerator kept at 0°C.

0.1566 gm. substance gave 0.3925 gm. CO_2 and 0.1622 gm. H_2O.

	Calculated for $C_9H_{18}O_2$:	Found:
C	68.29	68.35
H	11.47	11.59

7-Methyloctylic Amide.—The amide was prepared as described under 5-methylhexylic amide and was purified by crystallizing from alcohol. Melting point 106.5° (corrected).

0.1024 gm. substance neutralized 6.50 cc. 0.1 N HCl.

	Calculated for $C_9H_{19}NO$:	Found:
N	8.90	8.88

Ethyl 7-Methyloctylate.—The acid was esterified with alcohol and sulfuric acid in the usual manner. Another sample was esterified by saturating an alcoholic solution of the acid with hydrogen chloride. The yield of distilled ester in each case was about 90 per cent of the theory. 214 gm. of acid gave 228 gm. of ester. Boiling point 220.5°C. (corrected) at 764 mm. pressure.

0.1237 gm. substance gave 0.3208 gm. CO_2 and 0.1326 gm. H_2O.

	Calculated for $C_{11}H_{22}O_2$:	Found:
C	70.90	70.72
H	11.91	12.00

7-Methyloctyl Alcohol.—7-Methyloctylic ester was reduced in the manner described above. 125 gm. of ester gave 62 gm. of alcohol. 30 gm. of crude 7-methyloctylic acid were recovered. The yield of alcohol was 64 per cent. The yield taking the recovered acid into consideration is 89 per cent. Boiling point 206.0°C. (corrected) at 761 mm. pressure. The specific gravity at 25°C. is 0.8260 (1.6 cc. pyknometer was used).

0.1201 gm. substance gave 0.3292 gm. CO_2 and 0.1493 gm. H_2O.

	Calculated for $C_9H_{20}O$:	Found:
C	74.92	74.74
H	13.98	13.92

7-Methyloctyl Phenylurethane.—The urethane was prepared from 2 gm. of 7-methyloctyl alcohol and 1.8 gm. of phenylisocyanate. After three recrystallizations the melting point showed no further change. Melting point 66.4°C. (corrected).

0.2006 gm. substance neutralized 7.45 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{16}H_{25}NO_2$:	Found:
N	5.32	5.20

7-Methyloctyl Iodide.—The first lot of iodide was prepared by very slowly distilling the alcohol with five equivalents of constant boiling hydriodic acid. As the iodide did not distill over readily, in later preparations the acid and alcohol were refluxed for 4 hours and then the iodide was removed in a separatory funnel. The yield was 94 per cent of the theory. 35 gm. of alcohol gave 58 gm. of distilled iodide. Boiling point 120.0°C. (corrected) at 20 mm. pressure.

0.3055 gm. substance gave 0.2806 gm. AgI (Carius).

	Calculated for $C_9H_{19}I$:	Found:
I	49.90	49.65

Ethyl 7-Methyloctylmalonate.—71 gm. of 7-methyloctyl iodide were condensed with malonic ester in two lots. One and a half equivalents each of sodium and malonic ester were used to avoid as much as possible the formation of di-7-methyloctylmalonic ester. The yield was 65 per cent of the theory. Boiling point 182°C. (corrected) at 18 mm. pressure.[13]

0.1006 gm. substance gave 0.2480 gm. CO_2 and 0.0970 gm. H_2O.

	Calculated for $C_{16}H_{30}O_4$:	Found:
C	67.08	67.23
H	10.56	10.79

7-Methyloctylmalonic Acid.—The ester was saponified and the dibasic acid recovered from the soap in the usual manner. The very small yield of calcium salt from the mother liquors indicated that 7-methyloctylmalonic acid is practically insoluble in water. It crystallizes readily from benzene and is more soluble in petroleum ether than 5-methylhexylmalonic acid. Melting point 92°C. (corrected) without decomposition.

0.1055 gm. substance gave 0.2426 gm. CO_2 and 0.0930 gm. H_2O.

	Calculated for $C_{12}H_{22}O_4$:	Found:
C	62.56	62.70
H	9.63	9.86

9-Methyldecylic Acid.—57 gm. of 7-methyloctylmalonic acid were decomposed and the 9-methyldecylic acid was separated from alkali-insoluble impurities in the usual manner. The monobasic acid was then distilled three times under diminished pressure. It crystallizes in waxy plates at room temperature. It is slightly, if at all, volatile with steam. Boiling point 174.0–174.5°C. (corrected) at 23 mm. pressure.

0.1016 gm. substance gave 0.2650 gm. CO_2 and 0.1088 gm. H_2O.

	Calculated for $C_{11}H_{22}O_2$:	Found:
C	70.90	71.13
H	11.91	11.98

2-Methylpropyl Alcohol (Isobutyl Alcohol).—Two 1 kilo lots of Kahlbaum's isobutyl alcohol were used. They had no optical activity and when slowly distilled through a five bulb 18 inch column had a boiling point range of 106–108°. This alcohol was fractionated three times and the fraction boiling at 106–107° used.

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[12] It is recognized that to follow the homologous series closely this boiling point should be 2° higher or the boiling point of 6-methylhepthylmalonic ester should be lower. These two boiling points were, however, carefully checked several times. Over 40 gm. of substance were in each case available.

5 pounds of isobutyl alcohol prepared by the Du Pont Chemical Works were also used. This alcohol was purer than the Kahlbaum product. It had no optical activity and 85 per cent of it distilled at 106–107° on the first fractionation. This portion again fractionated through a five bulb column distilled completely at 106–107° (uncorrected).

4-Methylpentylic Acid.—Isobutyl iodide was prepared in the usual manner. All of the iodide was carefully fractionated through a five bulb column. That prepared from the Kahlbaum alcohol was found to contain some lower iodides but was obtained pure after three fractionations. The iodide prepared from the Du Pont alcohol was found to be very pure and was only fractionated once. The iodide used all boiled at 118–119° (uncorrected).

The iodide was condensed with malonic ester in 100 gm. lots. The isobutylmalonic ester was saponified in lots as large as 1 kilo in the manner described above. The soap was thoroughly extracted with acetone twice and then decomposed and the isobutylmalonic acid recovered in the usual manner. The large yield of calcium salt indicated that isobutyl malonic acid is readily soluble in water. As the calcium salt makes a 5 per cent solution in water the process is somewhat wasteful. The yield of crystalline isobutylmalonic acid was 50 per cent of the theory, calculated on the basis of the isobutyl iodide used. The dibasic acid was decomposed and the 4-methylpentylic acid freed from alkali-insoluble impurities in the usual manner. Boiling point, 199.2–199.7° (corrected). A sample of 4-methylpentylic acid prepared from 3-methylbutyl alcohol through the nitrile showed the same boiling point.

4-Methylpentyl Alcohol.—This alcohol has been prepared by several workers[14] and several different boiling points have been given for it.

4-Methylpentylic acid was esterified and the ester reduced in the usual manner. No record was kept of the yield of alcohol which was, however, known to be as good as in the other reductions. Through an oversight the alcohol was not analyzed. As the quantity prepared was large the boiling point given is probably correct. Boiling point 153°C. (corrected) at 760 mm. pressure.

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[14] Rossi, A., *Ann. Chem.*, 1865, cxxxiii, 180. Bouveault and Blanc, *Bull. Soc. chim.*, 1904, xxxi, 1210; *Chem. Zentr.*, 1905, 25. Buelens, A., *Bull. Acad. roy. Belg.*, 1908, 921; *Chem. Zentr.*, 1909, 832.

4-Methylpentyl Phenylurethane.—The urethane was prepared from 3 gm. of the alcohol in the usual manner. After six crystallizations from dilute alcohol its melting point showed no further change. Melting point 48°C. (corrected).

0.2010 gm. substance neutralized 9.07 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{13}H_{19}NO_2$:	Found:
N	6.33	6.32

4-Methylpentyl Iodide.—The iodide was prepared and purified in the usual manner. The yield was 68 per cent of the theory, not allowing for the iodide obtained by redistilling the low boiling portions of iodide with acid. Boiling point 173.2°C. (corrected) at 762 mm. pressure.

0.2087 gm. substance gave 0.2326 gm. AgI (Carius).

	Calculated for $C_6H_{13}I$:	Found:
I	59.95	60.25

Ethyl 4-Methylpentylmalonate.—The iodide was condensed with malonic ester in 50 gm. lots. A portion of the ester was purified and gave the following values. Boiling point 137.0°C. (corrected) at 11 mm. pressure.

0.0994 gm. substance gave 0.2320 gm. CO_2 and 0.0906 gm. H_2O.

	Calculated for $C_{13}H_{24}O_4$:	Found:
C	63.88	63.65
H	9.91	10.20

4-Methylpentylmalonic Acid.—The crude ester was saponified, the soap washed with acetone, and the acid recovered in the usual manner. The acid is moderately soluble in water, crystallizes well from benzene, and is quite insoluble in petroleum ether. A portion after three crystallizations from benzene showed no further change in melting point. Melting point 86.5°C. (corrected) without decomposition.

0.1280 gm. substance gave 0.2696 gm. CO_2 and 0.0996 gm. H_2O.

	Calculated for $C_9H_{16}O_4$:	Found:
C	57.41	57.44
H	8.57	8.71

6-Methylheptylic Acid.—The dibasic acid was decomposed and the monobasic acid freed from alkali-insoluble impurities in the usual manner. The 6-methylheptylic acid was then distilled twice under diminished pressure. Boiling point 126–127°C. (corrected) at 14 mm. pressure and 232°C. at 762 mm. pressure. It solidifies in a refrigerator kept at 0°C. A sample of the acid prepared from 5-methylhexyl iodide through the nitrile gave the same boiling point.

0.1500 gm. substance gave 0.3648 gm. CO_2 and 0.1478 gm. H_2O.

	Calculated for $C_8H_{16}O_2$:	Found:
C	66.58	66.32
H	11.18	11.04

6-Methylheptylic Amide.—8 gm. of 6-methylheptylic acid were converted into the acid chloride and then into the amide in the usual manner. The substance crystallized in white glistening plates. The constant melting point is 114.0°C. (corrected).

0.1002 gm. substance gave 0.2459 gm. CO_2 and 0.1068 gm. H_2O.

	Calculated for $C_8H_{17}ON$:	Found:
C	67.06	66.92
H	11.97	11.94

Ethyl 6-Methylheptylate.—The acid was esterified with 99.5 per cent alcohol and sulfuric acid. The ester was washed with water, dried, and distilled five times. Boiling point 200.3°C. (corrected) at 761 mm. pressure.

0.1394 gm. substance gave 0.3552 gm. CO_2 and 0.1473 gm. H_2O.

	Calculated for $C_{10}H_{20}O_2$:	Found:
C	69.70	69.50
H	11.71	11.83

6-Methylheptyl Alcohol.—6-Methylheptylic ester was reduced in the usual manner. Difficulty in purifying the alcohol brought the yield of distilled alcohol down to 80 per cent, taking into consideration the recovered acid. 144 gm. of ester gave 71 gm. of alcohol or 65 per cent of the theory. Boiling point 188.5°C. (corrected) at 764 mm. pressure. Specific gravity at 25°, 0.8230 (1.6 cc. pyknometer used).

0.1180 gm. substance gave 0.3179 gm. CO_2 and 0.1476 gm. H_2O.

	Calculated for $C_8H_{17}O$:	Found:
C	73.77	73.47
H	13.94	14.01

6-Methylheptyl Phenylurethane.—3 gm. of alcohol were converted into the urethane in the usual manner. After three crystallizations from alcohol the melting point showed no further change. Melting point 81.0–81.4°C. (corrected).

0.1006 gm. substance neutralized 4.05 cc. 0.1 N HCl (Kjeldahl).

	Calculated for $C_{15}H_{23}O_2N$:	Found:
N	5.62	5.64

6-Methylheptyl Iodide.—50 gm. of 6-methylheptyl alcohol were refluxed for 4 hours with five equivalents of constant boiling hydriodic acid. The yield of distilled iodide was 81 gm. or 88 per cent of the theory. It is insoluble in hydriodic acid and makes about a 20 per cent solution in absolute methyl alcohol. Boiling point 100.0°C. (corrected) at 17 mm. pressure.

0.1713 gm. substance gave 0.1671 gm. AgI (Carius).

	Calculated for $C_8H_{17}I$:	Found:
I	52.87	52.73

Ethyl 6-Methylheptylmalonate.—51.5 gm. of 6-methylheptyl iodide were condensed with malonic ester using one and a half equivalents each of sodium and malonic ester. The yield of distilled ester was 42.5 gm. or 73 per cent of the theory. Two distillations under diminished pressure gave a pure sample. Boiling point 171.8°C. at 18 mm. pressure. This boiling point was determined with unusual care.[13]

0.1039 gm. substance gave 0.2523 gm. CO_2 and 0.0950 gm. H_2O.

	Calculated for $C_{15}H_{28}O_4$:	Found:
C	66.12	66.22
H	10.36	10.24

6-Methylheptylmalonic Acid.—The distilled 6-methylheptylmalonic ester was saponified and the soap washed with acetone. The acid was recovered from the soap and recrystallized in the usual manner. It crystallizes well from benzene and is moderately soluble in petroleum ether. Melting point 89.5–90.0°C. (corrected) without decomposition.

0.1020 gm. substance gave 0.2279 gm. CO_2 and 0.0859 gm. H_2O.

	Calculated for $C_{11}H_{20}O_4$:	Found:
C	61.06	60.93
H	9.33	9.43

8-Methylnonylic Acid.—The 6-methylheptylmalonic acid was decomposed and the monobasic acid freed from alkali-insoluble impurities in the usual manner. The 8-methylnonylic acid was distilled three times under diminished pressure. After the first distillation the whole quantity was of practically constant boiling point. The acid solidifies at room temperature. Boiling point 155.6°C. (corrected) at 16 mm. pressure.

0.1242 gm. substance gave 0.3174 gm. CO_2 and 0.1296 gm. H_2O.

	Calculated for $C_{10}H_{20}O_2$:	Found:
C	69.70	69.68
H	11.71	11.69

Oxidation with Hydrogen Peroxide.

The acids employed for the oxidation experiments were in every case ester-free and of constant boiling point. The hydrogen peroxide varied in strength from 2.67 to 3.87 per cent as shown by titration. All the samples of hydrogen peroxide reacted acid to Congo red and contained traces of inorganic matter.

In alkaline solution the oxidations were not successful and little or no acetone was formed. A maximum yield of acetone was obtained when the oxidation was done in slightly acid solution. In those oxidations in which it was desired to obtain aldehydes the distillation was quite rapid. When a maximum yield of acetone was desired the rate of distillation was slower.

Small condensers were used to avoid mechanical loss of aldehydes and acids. The condensers extended below the surface of the liquid in the receivers which were very efficiently cooled with an ice mixture. By this means the loss of material was effectively prevented.

Two series of oxidations were carried out. The first series had for its purpose the preparation and identification of the aldehydes, volatile acids, and ketones. The second series aimed to show the effect on the yield of acetone of the increasing distance between the tertiary carbon atom and the carboxyl group. In this series the number of distillations was reduced to a minimum.

Preparation of Samples for Oxidation.—A given weight of the acid was mixed with about 10 cc. of water, approximately neutralized with 10 per cent sodium hydroxide, and the calculated amount of hydrogen peroxide added. The mixture was made neutral to phenolphthalein and then about 0.3 cc. of 10 per cent sulfuric acid added.

Procedure for the First Series of Oxidations.—The mixture of soap and hydrogen peroxide was distilled until the volume of the liquid in the flask was reduced to 20 to 30 cc. This residue contained the sodium salts of the unoxidized acid and of any acidic oxidation products, together with inorganic salts. It will be referred to as the oxidation residue.[15] If it showed the presence of formic acid (silver test) a few cc. of 0.5 N potassium permanganate solution were added and the mixture was placed in the refrigerator at 0°C. for a few hours. Following this it was acidified and the acids were extracted with ether in the customary way. Finally the acids were converted into silver salts.

Following the procedure of Dakin, the distillate from the oxidation was separated into two fractions, one containing ketones and aldehydes, and the other the volatile acids. In order to bring about this separation the distillate was rendered alkaline and redistilled. The residue contains the soaps of the volatile acids and the distillate contains the aldehydes and ketones. The volatile acid residue was treated in the same manner as the oxidation residue. In order to separate the aldehydes from the ketones they were oxidized by means of Tollens' reagent into the corresponding acids. After this the mixture was made acid with sulfuric acid and distilled nearly to dryness. This distillate again was rendered slightly alkaline and redistilled until the volume of the liquid in the distilling flask was reduced to 20 to 30 cc. The volatile acids of this final residue were identified in the form of silver salts. They will be referred to as acids from aldehydes. The distillate containing the ketones was extracted twice with ether. For identification of the acetone the extracted solution was treated with a solution of *p*-nitrophenylhydrazine. The reagent was prepared, according to the method of Dakin,[16] by dissolving one part of the hydrazine in thirty parts of hot 40 per cent acetic acid and then cooling and filtering the solution. The hydrazone which precipitated almost at once was filtered off and crystallized from alcohol until pure.

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[15] Several unsuccessful attempts were made to isolate dibasic acids from the oxidation residues from 4-methylpentylic and 5-methylhexylic acids. No indication of the presence of these acids was obtained.

Procedure for the Second Series of Oxidations.—3 gm. lots of acid were prepared for oxidation in slightly acid solution as described above. Three and a half equivalents of hydrogen peroxide were used. The mixture was distilled until the volume of liquid in the distilling flask was reduced to 20 to 30 cc. The aldehydes in the distillate were oxidized with Tollens' reagent. The mixture was then made acid with strong sulfuric acid and distilled almost to dryness. The new distillate which contains volatile acids and ketones was used for the estimation of acetone. It was separated into two parts and one part was employed for titration directly. The other part was extracted with one-fourth its volume of alcohol-free ether prior to titration. The object of this treatment was to remove the difficulty which was encountered in the titration of the ketone fraction obtained in the experiments with acids higher than isocaproic. The ketones formed as the result of β oxidation in these experiments, owing to their insolubility in water, make it difficult to obtain a uniform sample for titration. It was established by preliminary experiment that an aqueous solution of acetone of a concentration approaching that of our experiments loses under these fixed conditions between 11 and 14 per cent of the original acetone. It is recognized that in the case of the higher acids the accuracy of the data obtained for the unextracted solutions is open to question. Nevertheless the values reported are the result of titrations which check closely and the results are serviceable for comparison.

The acetone was titrated by the Messinger-Huppert iodine-thiosulfate method. It was found necessary to carry out the titration in a large and uniform volume of liquid to avoid the errors otherwise arising from the heat of neutralization of the 10 cc. of 40 per cent sodium hydroxide used in the process. 10 cc. lots of the distillates containing from 5 to 20 mg. of acetone were diluted to 400 cc. and the titration was carried out in the usual manner. 1 cc. of 0.1 N iodine is equivalent to 0.967 mg. of acetone.

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[16] Dakin, *J. Biol. Chem.*, 1908, iv, 235.

Oxidation of Isobutyric Acid (Second Series).—Kahlbaum's isobutyric acid was distilled twice and the fraction boiling at 155.2–155.5°C. (corrected) was used. The acid was oxidized in the manner described above and the following data were obtained.

TABLE II.

Total volume.	Volume titrated.	0.1 N I used.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
250	10	13.25	11.75	320	284
250	10	12.45	11.05	310	267
263	10	9.45	8.55	240	218

Another series of isobutyric acid oxidations was carried out to prove the formation of volatile acids of lower molecular weight. 5 gm. lots of isobutyric acid were prepared for oxidation in the usual manner. Only one equivalent of hydrogen peroxide was used. The distillates from the oxidations were made alkaline and redistilled. The residue from this distillation contains isobutyric acid and the lower acids formed by the oxidation. Silver salts were made from the residues and gave the following values. The absence of formic acid was indicated by these salts being entirely stable. The molecular weights were determined on the basis of the silver content.

Sample.	Silver.	Mol. Wt.	
		Found:	Calculated for $C_3H_5O_2Ag$:
mg.	*mg.*		
0.0559	0.0332	179.4	180.9
0.1233	0.0735	181.0	
0.1433	0.0815	189.9	

Oxidation of 3-Methylbutylic Acid (Second Series).—Pure 3-methylbutylic acid was prepared by the oxidation of 3-methylbutyl alcohol with sodium bichromate. The acid was oxidized in the manner described above. The following data were obtained.

TABLE III.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	21.2	18.8	410	364
215	10	20.5	17.7	426	368

Oxidation of 4-Methylpentylic Acid (First Series).—5 gm. lots of 4-methylpentylic acid were oxidized as described under the procedure for the first series of oxidations. Table IV gives the analytical data obtained.

TABLE IV.

Source of silver salt.	Sample.	Silver.	Mol. Wt.	
			Found:	Calculated for $C_6H_{11}O_2Ag$:
	mg.	*mg.*		
Oxidation residue.....	0.1794	0.0867	223.1	222.9
" "	0.1966	0.0951	223.1	
" "	0.1633	0.0784	224.7	
Volatile acids.........	0.2013	0.0965	225.2	222.9
" "	0.1861	0.0898	223.6	
				$C_5H_9O_2Ag$:
Acids from aldehydes.	0.1332	0.0697	206.2	208.9
" " "	0.1552	0.0816	205.3	
" " "	0.0131	0.0068	207.9	

The *p*-nitrophenylhydrazone of acetone obtained was crystallized twice from alcohol. Melting point 148.5–149.0°C (corrected).

0.1366 gm. substance gave 0.2798 gm. CO_2 and 0.0729 gm. H_2O.

	Calculated for $C_9H_{11}O_2N_3$:	Found:
C................................	55.95	55.85
H................................	5.74	5.97

Oxidation of 4-Methylpentylic Acid (Second Series).—The acid was oxidized as described under the procedure for the second series of oxidations. The following analytical data were obtained.

TABLE V.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	15.7	12.95	306	205
250	10	12.95	10.95	313	265
200	10	14.0	11.55	271	223

Oxidation of 5-Methylhexylic Acid (First Series).—Since a considerable amount of 5-methylhexylic acid was available the oxidations were carried out on larger quantities. The results show a normal increase in the yield of oxidation products. The oxidations were carried out as described under the procedure for the first series of oxidations. 5 gm. lots of acid were oxidized with five equivalents of hydrogen peroxide. Table VI gives the analytical data obtained.

TABLE VI.

Source of Ag salt.	Sample.	Silver.	Mol. Wt.	
			Found:	Calculated for $C_7H_{13}O_2Ag$:
	mg.	*mg.*		
Oxidation residue.....	0.1983	0.0904	236.7	236.9
" "	0.1624	0.0742	236.0	
Volatile acids.........	0.1002	0.0466	232.1	236.9
" "	0.1857	0.0847	236.5	
				$C_6H_{11}O_2Ag$:
Acids from aldehydes.	0.1613	0.0783	222.1	222.9
" " " .	0.2110	0.1017	223.8	

The *p*-nitrophenylhydrazone of acetone obtained was crystallized three times from alcohol. Melting point 149.2°C. (corrected).

Oxidation of 5-Methylhexylic Acid (Second Series).—The acid was oxidized as described under the procedure for the second series of oxidations. Table VII gives the analytical data obtained.

TABLE VII.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	12.45	9.25	241	179
200	10	12.55	9.25	243	179
200	10	17.30	13.05	335*	252*
250	10	14.25	10.65	344*	257*

* These oxidations were carried out under different conditions.

Oxidation of 6-Methylheptylic Acid (Second Series).—The acid was oxidized in the manner described above and the following analytical data were obtained.

TABLE VIII.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	11.5		222	
220	10	10.3		219	
213	10	12.45	9.45	256*	195*
210	10	12.65	9.3	257*	189*
200	10		8.25		159
200	10		7.70		149
160	10		9.2		142

* These oxidations were carried out under different conditions.

After titration all solutions were combined and extracted twice. The extracted solution was used for the preparation of the *p*-nitrophenylhydrazone of acetone. The hydrazone was crystallized three times from alcohol. Melting point 148.5–149.5°C. (corrected).

0.1003 gm. substance gave 0.2049 gm. CO_2 and 0.0535 gm. H_2O.

	Calculated for $C_9H_{11}N_3O_2$:	Found:
C	55.95	55.71
H	5.74	5.97

Oxidation of 7-Methyloctylic Acid (*Second Series*).—The acid was oxidized in the manner described above. Table IX gives the analytical data obtained.

TABLE IX.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	4.85	4.02	93	78
200	10	6.92	5.12	134	99

To secure a larger amount of acetone the oxidation residue left after the first distillation was oxidized twice more with three equivalents of hydrogen peroxide in the usual manner. All the distillates containing acetone were combined and extracted twice with ether. The solution was then distilled until about one-third of it had passed over. The new distillate was used for making the *p*-nitrophenylhydrazone. The residue from the distillation gave no test for acetone. The hydrazone was crystallized three times from alcohol. Melting point 148.5–149.5°C. (corrected).

Oxidation of 9-Methyldecylic Acid (*Second Series*).—The acid was oxidized in the manner described above. Table X gives the analytical data obtained.

TABLE X.

Total volume.	Volume titrated.	0.1 N I required.		Acetone.	
		Before extraction.	After extraction.	Before extraction.	After extraction.
cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
200	10	3.72	2.72	72	53
200	10	3.72	2.72	72	53

The oxidation residues were oxidized twice more and the acetone *p*-nitrophenylhydrazone was prepared as described under the oxidation of 7-methyloctylic acid. Only a small quantity of hydrazone was obtained. This was crystallized twice from alcohol. Melting point 146–148°C. (corrected).

TABLE XI.

Average of Data from the Second Series of Oxidations.

Acid.	Acetone.							Loss on extraction.
	Theoretical yield.	Before extraction.			After extraction.			
		Actual yield.	Per cent of theory.	Yield per gm. mol.	Actual yield.	Per cent of theory.	Yield per gm. mol.	
	mg.	*mg.*		*mg.*	*mg.*		*mg.*	*per cent*
2-Methylpropylic.	1,978	290	14.6	851	258	13.0	757	11.0
3-Methylbutylic...	1,707	418	24.5	1,421	366	21.4	1,245	12.4
4-Methylpentylic..	1,500	297	19.8	1,149	246	16.4	952	17.2
5-Methylhexylic ...	1,338	242	18.2	1,049	179	13.4	776	25.4
6-Methylheptylic ..	1,208	220	18.2	1,056	150	12.4	720	25.0*
7-Methyloctylic...	1,101	114	10.3	600	89	8.1	469	22.0
9-Methyldecylic....	936	72	7.7	447	53	5.7	311	26.4

* Calculated from the data marked with an asterisk under oxidation of 6-methylheptylic acid (Table VIII).

THE MECHANISM OF CHOLESTEROL ABSORPTION.

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By the analysis of chyle obtained from thoracic duct fistulas in dogs cholesterol which was fed has been shown[1] to be absorbed from the alimentary tract through the lymphatic system, whether fed as free cholesterol or as cholesterol esters. An increase in both fractions was always recognizable in the chyle, so that the normal proportion of one part of free to two or three parts of combined was quite constantly maintained. It seemed desirable to investigate further the mechanism of this process, particularly since, as has been pointed out,[2] there are rather important theoretical reasons for studying the process of a physiological interchange between free and combined cholesterol.

There are three general locations in which the esterification or saponification processes can take place during the absorption of cholesterol and its esters. *A priori* it might be expected from the general chemical and physical similarities between these bodies and the neutral fats that the same enzymes and tissues which are connected with the absorption of the latter must also take part in that of the former. The enzymes within the gastro-intestinal tract, the intestinal mucosa, and the mesenteric lymph nodes[3] all are concerned in the absorption of neutral fats. Cholesterol, during its absorption into the chyle of the thoracic duct, must be subjected to the activities of the same enzymes and tissues. The experiments to be described have been directed at all these agencies; and while it may be stated here that the results do not furnish sufficient evidence for a complete explanation of the changes which apparently take place, yet it is thought they may throw some little light on the process.

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[1] Mueller, J. H., *J. Biol. Chem.*, 1915, xxii, 1.

[2] Mueller, *J. Biol. Chem.*, 1916, xxv, 561.

[3] Stheeman, H. A., *Beitr. path. Anat. u. allg. Path.*, 1910, xlviii, 170.

Influence of the Gastro-Intestinal Tract on Cholesterol Absorption.

A possible change in the ingested cholesterol taking place in the stomach or intestine was sought for in three general ways: (1) by analysis of the gastric and intestinal contents after feeding cholesterol; (2) by following the absorption through a thoracic duct fistula with experimental elimination of certain of the digestive juices; and (3) by experiments *in vitro* dealing with the possible action of various enzymes upon cholesterol.

The determination of cholesterol was carried out by the Fraser and Gardner[4] modification of the Windaus[5] digitonin method. The extract of the material was prepared in the case of fluids, such as chyle, by the alcohol-ether method of Bloor,[6] and in the case of tissues, by treating the finely divided material with hot alcohol-ether followed by several extractives with boiling alcohol. To remove water-soluble extractives, which are always present, the combined extracts were evaporated to a small bulk, water and salt added (to prevent formation of emulsions), and the material extracted thoroughly with ether. The details of this method I have discussed elsewhere.[7]

Experiment 1.—After 24 hours' fasting, a dog was allowed to eat four raw eggs, shaken up in 350 cc. of water to which a little NaCl had been added. 10 cc. of this egg mixture were reserved for analysis. After 2 hours the stomach contents were removed by tube. A 20 cc. sample was analyzed. Peptic digestion had apparently been active, for the precipitate formed on adding alcohol to the digested sample was very light and fine compared to the heavy curdy precipitate of the fresh mixture.

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[4] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1910, lxxxii, 559.

[5] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[6] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

[7] Mueller, *J. Biol. Chem.*, 1916, xxv, 549.

	gm		
Sample 1.			
Free	0.0236		
Combined	0.0020	C : T*	7.8
Sample 2.			
Free	0.0251		
Combined	0.0027	C : T	9.7

* C : T denotes the ratio of combined cholesterol to total cholesterol on a percentage basis.

Experiment 2.—A dog was allowed to eat a small piece of meat and three raw eggs. 4 hours later it was killed, and the contents of the small and large intestines were removed separately. The small intestine contained about 150 cc. of thick yellow fluid, and the large intestine about 30 cc. of material of the same appearance, together with some dark colored solid feces. The latter were discarded. About 10 cc. of each of the contents of the large and small intestines were analyzed.

	gm.		
Small intestine.			
Free	0.0369		
Combined	0.0016	C : T	4.2
Large intestine.			
Free	0.0227		
Combined	0.0022	C : T	8.8

As far as can be seen from these results, no esterification of free cholesterol appears to take place in the stomach or intestine. It is quite possible, however, in spite of such negative findings, that the ester might be formed slowly and be absorbed practically as fast as formed.

Experiment 3.—A dog was fed bread and milk containing 10 cc. of sesame oil in which 1.25 gm. of cholesterol oleate had been dissolved. After killing the animal 3 hours later, at which time the mesenteric lymphatics were white and prominent, the gastric and intestinal contents were analyzed. The stomach contained a considerable amount of undigested bread mixed with drops of oil. An unweighed portion of this was extracted and analyzed. Only about 5 cc. of a thick, bile-stained fluid could be obtained from the small intestine; this was extracted.

	gm.		
Stomach.			
Free	0.0139		
Combined	0.0256	F : T*	35
Intestine.			
Free	0.0360		
Combined	0.0077	F : T	82

* F: T denotes the ratio of free cholesterol to total cholesterol on a percentage basis.

These figures seem to indicate that hydrolysis of the ester takes place in the intestine. In view of experiments to be described later, however, this interpretation is not so probable, and it is well to note that if there were a selective absorption of the esters from the intestine, the result would be the same. Possibly only a part of the meal had reached the intestine, for while the whole of the intestinal contents was analyzed, the total cholesterol amounting only to some 0.04 gm., only a part, probably less than one-tenth, of the stomach contents was used, so that there was present in the stomach perhaps 0.5 gm. of cholesterol. Since a considerable part of the cholesterol present in the intestine must have come from the bile, evidently there was too little ingested cholesterol present to warrant any definite conclusions from this experiment.

By studying the absorption of cholesterol through the thoracic duct after experimentally diverting a single secretion, such as bile or pancreatic juice, from the intestine, it was hoped to gain an insight into the part played by such enzymes in the process.

Experiment 4.—Immediately after feeding a dog a small piece of meat, the animal was etherized, a thoracic duct fistula produced, and a sample of chyle collected. Then, through a laparotomy incision, 10 cc. of a solution of egg white, containing a small amount of dilute hydrochloric acid, were injected by means of a large hypodermic needle, directly into the duodenum. It was hoped in this way to start the normal digestive secretions into the intestine. This injection was followed by 10 cc. of cottonseed oil, containing 2 gm. of cholesterol oleate, given in the same way. The dog recovered promptly from the ether. After 2 hours chyle was obtained which was only moderately cloudy, and 200 cc. of milk were given the dog. In 2 hours more chyle which was very milky was collected and analyzed.

		Free.	Combined.	F : T.
		per cent	*per cent*	
11.00 a.m.	Thoracic duct fistula completed. Chyle collected................	0.022	0.038	36
11.30 "	Oil injected into intestine.			
1.30 p.m.	Chyle collected, moderately milky. Fed 200 cc. of milk.			
3.30 "	Chyle collected, very milky.			
5.30 "	" " " "	0.065	0.185	26

That gastric digestion is not an essential feature in the absorption of cholesterol is shown by the results of this experiment. Perfectly normal, if somewhat delayed, absorption took place when the solution of cholesterol in oil was injected directly into the duodenum. The delay probably resulted from a failure of the hydrochloric acid injection to bring about the pancreatic secretion, so that only after starting the normal sequence by feeding a little milk was the intestinal digestion properly started.

In the experiments now to be described, in which the bile was excluded from the intestine, the same general plan was followed in each case. The common bile duct of the dog was ligated, under ether, the gall bladder was brought up into the wound, a rubber tube, with a projection on its inner end formed by rolling it back upon itself, was fastened into the gall bladder by a purse string suture, and the wound was closed. Bile was removed three or four times a day through this cannula; at other times it was closed by means of a small clamp and protected by a close fitting oilcloth coat. In two instances the dogs became somewhat jaundiced on the following day, once due to hemorrhage into the gall bladder, with subsequent clotting and occlusion of the cystic duct; the cause for the other was not determined. Thoracic duct fistulas were made on the 2nd day after the biliary fistulas in order to give time for the bile present in the intestine to be eliminated as far as possible. Feces passed by the dogs during the course of the experiment were clay-colored, indicating the absence of bile.

Experiment 5.

		Free.	Combined.	F : T.
		per cent	*per cent*	
Dec. 30.	Biliary fistula made.			
Jan. 1.				
11.30 a.m.	Thoracic duct fistula completed. Chyle taken	0.021	0.028	43
1.30 p.m.	Chyle taken. Fed small piece of meat.			
1.45 "	Fed four raw eggs.			
5.00 "	Chyle taken, very milky	0.056	0.038	60
6.00 "	" " " "	0.058	0.037	61

Experiment 6.

Jan. 17.	Biliary fistula made.			
" 19.				
11.15 a.m.	Thoracic duct fistula completed. Chyle taken	0.020	0.037	35
5.15 p.m.	Fed three raw eggs.			
8.15 "	Chyle taken, very milky	0.054	0.050	52
9.15 "	" " " "	0.050	0.037	57

Experiment 7.

Jan. 29.	Biliary fistula made.			
" 30.	Dog jaundiced (gall bladder contained clotted blood).			
Feb. 1.				
11.00 a.m.	Thoracic duct fistula completed. Chyle taken	0.037	0.044	46
12.00 "	Fed small piece of meat and three raw eggs.			
4.00 p.m.	Chyle taken, milky	0.051	0.063	45

Experiment 8.

Mar. 4.	Biliary fistula made.			
" 6.				
12.00 m.	Thoracic duct fistula completed.			
2.15 p.m.	Chyle taken. Fed four eggs.	0.022	0.045	33
5.15 "	Chyle collected, milky.	0.034	0.065	34
6.15 "	" " "	0.035	0.042	45

Experiment 9.

		Free.	Combined.	F : T.
		per cent	*per cent*	
June 6.	Biliary fistula made.			
“ 8.				
11.30 a.m.	Thoracic fistula completed. Chyle taken..................	0.047	0.058	44
2.00 p.m.	Chyle taken. Given 200 cc. of milk and three eggs...........	0.045	0.058	44
5.30 “	Chyle taken, very milky........	0.054	0.060	47
8.15 “	“ “ “ “	0.050	0.055	48

Experiment 10.

June 10.	Biliary fistula made.			
“ 12.				
10.00 a.m.	Thoracic duct fistula completed.			
2.30 p.m.	Given 200 cc. of milk. Chyle collected......................	0.057	0.094	38
	Fed 1.5 gm. of cholesterol oleate in 10 cc. of olive oil and 200 cc. of water.			
8.45 “	Chyle collected, quite milky..	0.073	0.087	46
	Fed 100 cc. of milk and then 1.5 gm. of cholesterol oleate in 10 cc. of cottonseed oil and 200 cc. of water, plus 3 gm. of dried ox bile.			
11.30 “	Chyle collected, quite milky.....	0.063	0.093	41

Experiment 11.

June 12.	Biliary fistula made.			
“ 14.				
10.00 a.m.	Thoracic duct fistula completed.			
1.00 p.m.	Chyle taken. Fed 1 gm. of cholesterol in 10 cc. of cottonseed oil and 200 cc. of milk....	0.038	0.066	36
5.00 “	Chyle taken. Fed 150 cc. of milk and then 1 gm. of cholesterol in 10 cc. of cottonseed oil and 200 cc. of water, plus 1 gm. of sodium taurocholate (Merck)..	0.041	0.065	39
9.00 “	Chyle taken, quite milky........	0.057	0.063	48
June 15.				
10.00 a.m.	“ “ “ “	0.062	0.083	43

The bile apparently has a rather important function in connection with cholesterol absorption. Rothschild states[8] that after tying the common bile duct in dogs no cholesterol is absorbed. This evidently does not hold strictly. It each case there is a rise in the total cholesterol after feeding. This rise is small in some cases, and quite pronounced in others, but in no case is it so pronounced as that obtained in normal dogs. It does not depend upon the amount of fatty material absorbed, for in all cases, after feeding, chyle was obtained which was quite milky, the alcohol-ether extracts of which left a considerable amount of oily material after evaporation.

It has been suggested that bile aids the absorption of neutral fats by the reaction of the bile acids with the fatty acids, and in this way makes possible the entrance of the water-insoluble fatty substance into the cells of the mucosa. Cholesterol is also held in solution by bile acids, and its absorption may be facilitated in a similar manner.

The other noticeable fact brought out by the experiments is the relative increase in free cholesterol absorbed. In each case the percentage of free to total cholesterol was above that found normally after feeding. This figure seems to vary normally between perhaps 25 and 35 per cent, while in the biliary fistula animals it was between 39 and 61 per cent. The significance of this variation is not evident.

Normal absorption was not produced by the addition of bile or sodium taurocholate to the diet.

The experiments in which the pancreatic juice was excluded from the intestine were planned in the same way, allowing at least 2 days after the first operation for the dog to recover as far as possible. Under ether, the two pancreatic ducts were ligated and cut, and the pancreas itself, while left *in situ*, was separated from the intestine throughout its entire course, except for the larger blood vessels. In this way, it was believed, only the intestinal secretion would be eliminated, while the far reaching disturbances in carbohydrate metabolism, incident upon a complete removal of the pancreas, would be obviated. All the dogs recovered rapidly from the first operation, and were apparently in good condition when the thoracic duct fistulas were done.

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[8] Rothschild, M. A., *Proc. N. Y. Path. Soc.*, 1914, xiv, 229.

Experiment 12.

		Free.	Combined.	F : T.
		per cent	*per cent*	
May 23.	Pancreatic ducts ligated and cut.			
" 27.				
11.00 a.m.	Thoracic duct fistula completed. Chyle taken	0.050	0.085	37
2.00 p.m.	Chyle taken. Fed 200 cc. of milk and two eggs.			
4.30 "	Chyle taken	0.051	0.074	41
6.00 "	" "	0.058	0.077	43
8.00 "	" "	0.058	0.080	42

Experiment 13.

May 24.	Pancreatic ducts ligated and cut.			
" 27.				
12.00 m.	Thoracic duct fistula completed. Chyle taken	0.047	0.084	36
2.00 p.m.	Chyle taken. Fed 2 gm. of cholesterol oleate in 10 cc. of olive oil and the white of an egg in 150 cc. of water.			
4.30 "	Chyle taken, milky	0.047	0.075	39
6.00 "	" " "	0.053	0.077	41
8.30 "	" " "	0.057	0.074	43

Experiment 14.

May 29.	Pancreatic ducts ligated and cut.			
June 1.				
12.00 m.	Thoracic duct fistula completed. Chyle taken.			
2.45 p.m.	Chyle taken. Fed 150 cc. of milk and then 2 gm. of cholesterol oleate in 10 cc. of olive oil, in 150 cc. of water containing the white of an egg.	0.050	0.104	33
5.00 "	Chyle taken	0.045	0.082	36
8.15 "	" " quite milky	0.057	0.083	41

Absence of the pancreatic juice seems to diminish the absorption of cholesterol very greatly. There is a slight increase in the free cholesterol after eating, but the cholesterol esters seem to decrease to some extent. The total cholesterol present remains nearly constant, but the ratio of free to total cholesterol rises somewhat. Here again, as in the case of the bile experiments, the decreased absorption cannot be due entirely to the absence of other lipoids in the chyle, for some of the samples at least were quite milky, although none contained as much fat as is present in normal digestion.

It seems evident that the pancreatic juice, and to a less extent the bile, exert an important influence on the absorption of cholesterol. If there could be demonstrated either a well marked hydrolytic action upon cholesterol esters, or an esterification of free cholesterol by one of the intestinal enzymes, it might help clear the situation. Experiments with this probability in view were undertaken with pancreatic extract, as well as with the intestinal mucosa of the dog. In the case of the pancreas, the organ was put through a meat chopper, and two or three parts of 50 per cent glycerol were added, to prevent as far as possible the activation of trypsin with the possible destruction of a lipase or "cholesterase." This suspension of hashed gland in dilute glycerol was used without filtering. The intestinal mucosa, after being scraped from the small intestine, was suspended in four parts of water. Since both cholesterol and cholesterol oleate are insoluble in aqueous media and solid at incubator temperature, a solution in sesame oil was used. This not only enabled the cholesterol to be presented to the enzyme action in a finely divided form, but also furnished an indication of ordinary lipase activity from the amount of fatty acid set free, which was estimated by titration.

For most of the experiments, four equal portions of 3 to 5 gm. of the ground pancreas or intestinal mucosa were taken, glycerol or water added to each, and then to each of one pair a small amount of sesame oil containing some free cholesterol, and to each of the other pair, some sesame oil containing cholesterol oleate, and, in some cases, 1 cc. of dog bile, were added. One of each pair was then mixed with alcohol-ether, and the other, after the addition of a few drops of toluene, incubated from 1 to 6 days, with occasional shaking, and then extracted with alcohol-ether and boiling alcohol. The ether extract finally obtained was divided into two parts as usual, and the part intended for determination of total cholesterol was first titrated in alcohol solution with alcoholic KOH. In each case, as will be seen, there was quite a marked increase in fatty acids, an evidence of good lipase action. If the neutral fat-splitting enzymes could also split cholesterol esters, such action should be apparent here. On the other hand, with the gradual formation of fatty acids, there was abundant opportunity for esterification of the free cholesterol, if such a process could take place under these conditions.

Experiment 15. Pancreas plus Bile.—5 gm. portions of pancreas were used, and 0.4 cc. of sesame oil with, respectively, 0.01 and 0.013 gm. of cholesterol and cholesterol oleate. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.01 gm., fresh........	3.0	0.0115	0.0007	94
" " 0.01 " incubated....	14.0	0.0042	0.0042	50
Cholesterol oleate 0.013 gm., fresh.....	3.9	0.0074	0.0047	61
" " 0.013 " incubated.	3.7	0.0015	0.0017	47

Experiment 16. Pancreas plus Bile.—5 gm. portions of pancreas were used, and to each of one pair was added 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to the other about 0.04 gm. of cholesterol oleate in 1.0 cc. of sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	3.8	0.0212	0.0006	97
" " 0.02 " incubated...	14.8	0.0104	0.0102	50
Cholesterol oleate 0.04 gm., fresh......	3.5	0.0062	0.0058	52
" " 0.04 " incubated.	17.0	0.0032	0.0100	24

Experiment 17. Pancreas plus Bile.—4 gm. portions were used, and to each of one pair was added 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to the other, 0.03 gm. of cholesterol oleate in 0.8 cc. of sesame oil. Incubated 6 days.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	Lost.	0.0282	0.0008	97
" " 0.02 " incubated...	10.0	0.0165	0.0120	58
Cholesterol oleate 0.03 gm., fresh......	Lost.	0.0123	0.0136	47
" " 0.03 " incubated.	10.8	0.0090	0.0131	41

Experiment 18. Pancreas Alone.—4 gm. portions of pancreas were used. To each was added 0.02 gm. of cholesterol in 0.08 cc. of sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	4.6	0.0270	0.0000	100
" " 0.02 " incubated...	13.4	0.0210	0.0109	66

Experiment 19. Intestinal Mucosa plus Bile.—5 gm. portions were used. To each was added 1 cc. of dog bile, and to each of one pair, a solution of 0.02 gm. of cholesterol in 0.8 cc. of sesame oil, and to each of the other two, 0.03 gm. of cholesterol oleate in sesame oil. Incubated 24 hours.

	Titration.	Cholesterol.		F: T.
		Free.	Combined.	
	cc.	*gm.*	*gm.*	
Free cholesterol 0.02 gm., fresh........	1.0	0.0284	0.0022	93
" " 0.02 " incubated...	13.2	0.0270	0.0030	90
Cholesterol oleate 0.03 gm., fresh......	1.0	0.0136	0.0162	47
" " 0.03 " incubated.	11.4	0.0130	0.0142	48

In none of these experiments was there any indication of a hydrolytic effect on the cholesterol oleate, in spite of the lipase action which occurred. On the other hand, in all experiments in which the pancreas was used, a rather striking esterification of the free cholesterol occurred. This was greatest in the experiments in which bile was used, but perfectly definite, also, in the fourth, in which there was only pancreas. In Experiment 1, the decreased amount of cholesterol found in the incubated samples is doubtless due to incomplete extraction. In the experiment with intestinal mucosa, there was no change demonstrable in either the free cholesterol or the oleate.

A synthetic action of this kind is not the rule for enzymes. True, a reversible effect can usually be demonstrated for most enzymes when conditions are carefully controlled. Here, however, the reaction apparently does not reach an equilibrium unless the greater part of the cholesterol has been esterified, since the samples to which cholesterol oleate had been added, and which contained only a relatively small amount of free cholesterol, also gave evidence of an esterification. On the other hand, since an enzyme is a catalytic agent which hastens a reaction that would take place slowly under ordinary conditions, it is not so unlikely that this should be a true enzyme action. Free cholesterol, as is well known, possesses strong inhibiting powers toward hemolytic substances of various kinds. It is not impossible that one of its functions is to combine with and render nontoxic, fatty acids which may become liberated in the body. Apparently it will combine very slowly with fatty acids set free in the brain after death.[9, 10] A raw egg mixed with water, and allowed to autolyze with tricresol and toluene for 6 months showed only 62 per cent of its total cholesterol to be free, as against about 90 per cent at the beginning. Seemingly there is a tendency for free cholesterol to unite slowly with fatty acids to form esters. It is quite conceivable that a body enzyme should hasten such a synthesis.

It is, of course, absolutely essential to show that this esterification did not take place during the extraction and analysis, from heating. The esters are prepared artificially by heating together the cholesterol and an excess of fatty acid to 200°. Possibly the evaporation on the water bath might have been enough to cause the change. Fortunately, the intestinal mucosa experiment, run parallel with those of the pancreas, acts as an

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[9] Mair, W., *J. Path. and Bact.*, 1913-14, xviii, 179.

[10] Lapworth, A., and Royle, F. A., *J. Path. and Bact.*, 1914-15, xix, 474.

excellent control. In addition, the effect of evaporating a mixture of oil, fatty acid, and free cholesterol on the water bath was tried. About 0.01 gm. of cholesterol, 0.4 cc. of sesame oil, and 0.4 cc. of fatty acid from saponification of sesame oil were mixed with about 25 cc. of alcohol and 1 or 2 cc. of water, and evaporated down on a water bath. The process was then repeated and the residue allowed to heat for several minutes to 100° after the water and alcohol had evaporated. Analysis of this mixture and of a duplicate unheated mixture showed 0.0130 gm. of free cholesterol before heating, and 0.0131 gm. after heating. In other words, no esters had been formed.

As a further precaution to prevent any possibility of the esterification taking place from heating, the extraction of Experiments 3 and 4 was carried out with as little heating as possible, the extracts concentrated at room temperature before an electric fan, taken up in water, extracted by ether, the ether evaporated until the temperature rose to 45–50°, and then the fatty acids present accurately neutralized with alcoholic KOH, using phenolphthalein as indicator. The solution, after diluting with water, was again extracted with ether, and this solution, free from fatty acids, was analyzed as usual. No difference in results is apparent between these two experiments and the others.

From these experiments it seems fair to conclude that there is in the pancreas some substance which accelerates the formation of cholesterol esters and that possibly the bile increases this effect. It may well be that this action of the pancreas is of fundamental importance in the absorption of cholesterol and that here is to be found the explanation for the failure of cholesterol to be absorbed into the chyle after ligation of the pancreatic ducts.

Influence of the Intestinal Mucosa on Cholesterol Absorption.

In order to study the possible effect of the intestinal mucosa on the absorption of cholesterol, two methods were utilized. The first, an experiment *in vitro* with ground mucosa, already described, gave no evidence of any activity. The second was quite definite in its results. Portions of intestinal mucosa were analyzed during fasting of 24 hours' duration, and during the active absorption of cholesterol or its esters. It was thought that with the rather considerable amount of fat present in the cells and lymph spaces of the mucosa, enough absorbed cholesterol might be present to be recognizable on analysis. In such experiments, digestion was allowed to proceed for 3 hours, and then the dogs were quickly killed by an intravenous injection of chloroform, the intestine was removed, opened, and washed as free as possible from adherent bile-stained mucus, and the mucosa scraped off with a dull knife. In the same way the material was obtained from dogs which had first been fasted for 24 hours.

The mucosa used for analysis was not carefully weighed out, and the figures are therefore given in grams of cholesterol found, rather than in percentage, and represent portions of mucosa of about 5 gm. weight.

	Duodenum.			Jejunum.			Colon.		
	Free.	Ester.	C: T.	Free.	Ester.	C: T.	Free.	Ester.	C: T.
	gm.	*gm.*		*gm.*	*gm.*		*gm.*	*gm.*	
24 hrs. fasting.....	0.0109*	None.	—						
24 " "	0.0092*	0.0006	6						
24 " "	0.0131	None.	—	0.0098	None.	—	0.0116	0.0006	4.9
Fed three eggs.....	0.0101	0.0021	17						
Fed 300 cc. of milk with 2 gm. of cholesterol in 8 cc. of cottonseed oil.	0.0111	0.0017	13						
Fed three eggs.....	0.0194	0.0061	24	0.0215	0.0064	23	0.0131	0.0014	9.7
Fed two eggs......	0.0079	0.0014	15	0.0105	0.0012	10	0.0071	0.0002	2.7

* A mixture of duodenum and jejunum mucosa analyzed.

In all the dogs which had been fasted, as well as in the mucosa of the large intestine of the others, there is comparatively little combined cholesterol, in some cases none, and in the others very little. On the other hand, the mucosa of the small intestine of all the dogs which had been fed showed considerably more cholesterol, the amounts ranging from 10 to 23 per cent of the total.

Evidently the cholesterol which is absorbed from the intestine has already been largely esterified by the time it reaches, or at least before it leaves, the lymphatics of the intestinal mucosa.

This fact of itself does not show whether the esters were formed in the intestinal canal and absorbed as such, or whether they were synthesized by the cells of the intestinal mucosa. Taken in connection with the work *in vitro* on the pancreas and intestinal mucosa, however, it seems very probable that the change takes place slowly in the intestine, and that an absorption of the esters takes place as rapidly as they are formed.

No striking difference in the direction of a higher percentage of free cholesterol is apparent in these experiments in which cholesterol was fed. Such an increase, if it existed, could not be recognized with any certainty even by the average results of a considerable number of experiments, for it evidently could not be very great, and there seems to be considerable individual variation in the normal cholesterol content of the intestinal mucosa.

Influence of the Mesenteric Lymph Nodes on Cholesterol Absorption.

No satisfactory experiment could be devised to show a possible effect of the mesenteric lymph nodes on the absorbed cholesterol. It was first attempted to destroy the lymphoid tissue of the body by massive doses of x-ray. A rather small dog was exposed to the rays for about half an hour each day for 5 successive days, during which time the small lymphocytes in the blood fell from 26 to 3 per cent.[11] A thoracic duct fistula was then made, and an analysis of the chyle obtained before and after feeding eggs showed a perfectly normal absorption. After completing the experiment the dog was killed, and the mesenteric lymph nodes, the thymus, and the spleen were examined. The thymus was extremely atrophic, although the dog was quite young. The spleen was rather small and firm, but lymphoid follicles were visible in the gross. The lymph nodes were normal in the gross, but microscopically the germinal centers were somewhat small, and the pulp seemed to contain much less than the usual number of lymphoid cells. But the destruction was by no means sufficient to make it unlikely that they would exercise a normal function on the fat and lipoids passing through them in the chyle.

An attempt to demonstrate the presence of an enzyme in the gland by a technique similar to that used in the case of the pancreas and the intestinal mucosa also gave negative results. Fatty acids were produced by the lipase of the glands, and the experiments may thus also serve as controls on the earlier experiments with pancreas.

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[11] I am indebted to Dr. M. J. Sittenfield for raying the animal for me.

	Titration.	Free.	Combined.	F: T.
	cc.	gm.	gm.	
Free cholesterol, fresh............	0.9	0.0163	0.0002	99
" " incubated........	5.0	0.0136	0.0006	96
Cholesterol oleate, fresh...........	0.5	0.0047	0.0037	56
" " incubated......	1.3	0.0036	0.0028	56

In none of the experiments is there any indication either of a saponification of the esters, or of an esterification of the free cholesterol. The lower values found in the fourth are doubtless due to incomplete extraction, for here again it was attempted to avoid an accidental esterification of cholesterol through heating with the fatty acids formed, by the procedure previously described of extracting with no more heat than necessary, and subsequently evaporating before an electric fan, instead of distilling, and neutralizing the fatty acids present.

DISCUSSION.

Of the experimental data here presented there are only a few facts which stand out as definite. First, the bile, and even to a greater extent the pancreatic secretions, seem intimately connected with cholesterol absorption, indeed to a degree somewhat out of proportion to their influence on neutral fat absorption. By experiments *in vitro* it was shown that free cholesterol, in the presence of fatty acids and a suspension of pancreas, undergoes esterification, and from control experiments it seems most likely that this is a real action by the pancreas. In such experiments the addition of a little bile seems to make the change somewhat greater, but not enough experiments of this kind were done to make any generalizations.

By analysis of the intestinal mucosa during starvation and after feeding it was shown that an esterification of at least a large part of the absorbed cholesterol had already taken place.

Since no effect of the mucosa could be demonstrated *in vitro*, it is at least possible that the esterification may take place in the lumen of the intestine under the influence of the pancreatic juice, and that the esters may be absorbed as rapidly as formed.

Again, no evidence that the mesenteric lymph nodes take any part in cholesterol absorption could be obtained. The evidence of their part in neutral fat absorption is, as far as I am aware, based on morphology and differential fat staining, although lymphoid tissue undoubtedly contains an active lipase.

One fact which stands out prominently is the resistance of cholesterol esters to saponification by ordinary active lipases. In no case has there been the slightest evidence of splitting, although neutral fat in the same mixture was easily saponified.

Taken altogether the data here presented by no means explain the whole process of cholesterol absorption. The peculiar effect of the bile in altering the proportion of free cholesterol absorbed is entirely unexplained. More important yet, while the origin of the cholesterol esters seen in the chyle of normal animals after feeding cholesterol-rich meals has been at least in part explained, the increase in free cholesterol and the mechanism which regulates the proportion of the two and keeps it constant has not been cleared up at all. However, the inherent difficulties encountered in carrying out the work make it appear quite useless to seek a more complete understanding of the process by means of the methods at present available.

I am indebted to Dr. W. G. MacCallum and Dr. A. M. Pappenheimer for assistance and suggestions in planning and carrying out the experiments, and to Dr. L. B. Mendel for helpful criticisms in the preparation of the paper.

PANCREATIC DIABETES IN THE DOG.

I. THE INFLUENCE OF ALKALI AND ACID UPON THE GLYCOSURIA AND HYPERGLYCEMIA.

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INTRODUCTION.

While controlling the influence of Knowlton and Starling's extract on the metabolism of depancreatized dogs, the writers reported (1) the observation that in a completely depancreatized dog the intravenous injection of 150 cc. of a 1 per cent solution of sodium carbonate in Ringer's solution lowered the G: N ratio from 3.07 to 0.88 within less than 3 hours after the injection was discontinued. A glance at Table I, repeated from the previous paper, shows that the fall in the ratio began at once but within the first 2 hours was due to a rise in the nitrogen output. During the second 2 hours the nitrogen excretion reached its previous level, while the sugar elimination dropped from 0.823 to 0.233 gm. per hour. The sugar per hour then began to rise, but even after 6 hours it had risen to but little more than half of what it was in the period preceding the injection.

TABLE I.

Date.	Time.	Total glucose.	Total nitrogen.	Glucose per hr.	Nitrogen per hr.	G: N.	Volume of urine.
1913		*gm.*	*gm.*	*gm.*	*gm.*		*cc.*
Apr. 24	2.19-4.19 p.m.	1.647	0.519	0.823	0.269	3.07	22
	4.26-5.15 "	150 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.					
	4.19-6.19 "	1.647	0.644	0.823	0.322	2.55	60
	6.19-8.19 "	0.466	0.524	0.233	0.262	0.88	24
" 25	1.19 a.m.	2.413	1.475	0.480	0.295	1.62	90

A search of the literature revealed the fact that in 1910 Pavy and Godden (2) administered a 3 per cent solution of anhydrous sodium carbonate to cats in which they had induced glycosuria by the administration of chloroform, and observed a fall in the intensity of the glycosuria within 15 minutes after the intravenous injection was begun, the urine becoming sugar-free at the end of 30 minutes. Pavy and Bywaters (3) had previously found that the postmortem production of sugar in the liver may be virtually checked by the influence of sodium carbonate injected in a 5 per cent solution (and even less strength) into the portal system of the living animal. On the other hand, the addition of rising quantities of acetic acid to the liver thus treated brought about an increase in enzymic power, and hence increased glycogenolysis, until a certain optimum was reached. They assumed that the inhalation of chloroform produced a condition of acidosis which led to hyperglycogenolysis and ultimately to glycosuria. The authors referred to seem to have studied the subject no further. They did not attempt to show whether sodium carbonate would exert the same effect in other forms of glycosuria. More recently Elias and Kolb (4) studied the influence of sodium carbonate upon glycosuria, hyperglycemia, and upon sugar tolerance in "hunger diabetes." They conclude from their observations that this alkali reduces the glycosuria and glycemia by influencing the intermediary metabolism of carbohydrates in the liver.

These facts clearly suggest the possibility that administration of alkali to diabetics may be of importance in more ways than one. The express object of alkali therapy is control of the acidosis, but it is possible that in so restoring the balance of ions in the tissues, the glycogenic functions and even the combustion of glucose may be assisted. The chance observation made while controlling the effect of Knowlton and Starling's extract of pancreas (5) has been followed up now for more than 3 years in this laboratory and we have accumulated much evidence that the regulation of acid and basic radicals and possibly the regulation of the kations amongst themselves is an important factor in preserving the capacity to oxidize glucose. A full discussion of the bearing of these facts will be deferred until the facts themselves are in evidence.

In this paper will be presented the facts first developed in our study of the influence of sodium carbonate upon the metabolism of the dog after pancreatectomy, as far as they relate to urine and blood. Coincidentally with these observations, some preliminary data were obtained regarding the respiratory metabolism, but it will be more convenient to present the evidence from this quarter *in extenso* separately.

Methods of Experimentation.

Female dogs large enough to permit of passage of a catheter with the help of a speculum have been used exclusively and the urine has in nearly every instance been so separated in definite periods. The bladder was always irrigated with saturated boric acid solution at the close of each washing. The urines have been most carefully preserved, as a rule with 10 per cent thymol in toluene (though in some instances with concentrated sulfuric acid) and refrigeration to 5°C.

Blood has been drawn, both with the needle by venapuncture, and after insertion of a cannula under local anesthesia. We prefer the latter method and in our later experiments have followed it almost exclusively because of the greater precision with which a sample can be taken at a desired time. No difficulty has been experienced on account of the "emotional glycosuria" of Cannon, Shohl, and Wright (6). However, while Scott (7), Seelig (8), and Rolly and Oppermann (9) do not believe that the dog is subject to emotional *glycosuria,* Loewy and Rosenberg (10) have shown that pain or fright incident to drawing blood may increase the *glycemia* to quite abnormal figures, and Shaffer (11) presents evidence to the same effect. We have, therefore, always exercised great care to avoid the causation of pain and to keep the animal perfectly quiet for some time preceding the opening of a vessel.

In most instances the blood has been drawn into a Brodie pipette by displacement of mercury, the blood mixing as it enters with 0.3 cc. of leech extract (hirudin). We agree with Epstein and Baehr (12) as to the importance in comparative analyses of knowing whether the blood has itself undergone a change of concentration and, as a means of control, we have made many determinations of the total specific gravity by first measuring accurately 1 or 2 cc. of blood into a weighed flask and immediately weighing the sample. Satisfactory duplicates of the specific gravity have nearly always been obtained in this way.

There are obvious advantages in the use of the specific gravity rather than the ratio of blood plasma to corpuscles as determined by means of the hematocrit. Hamburger and others have shown that the blood corpuscles change considerably in volume with changes in the concentration of the plasma and, in fact, the most common use of the hematocrit is for the demonstration of such changes in volume of the corpuscles rather than for demonstration of a change in concentration of plasma (13). Moreover, since the blood sugar is found in the corpuscles as well as in the plasma, it is difficult to see how a knowledge of the relation of plasma to corpuscles helps to give the correct percentage of blood sugar. A blood count would be a better index.

The method of Lewis and Benedict (14), either as originally reported, or as published in final form, has been used throughout for the blood sugar, and the method of Benedict for the urinary sugar.

Control of the Effect of Alkali on the Sugar in the Urine.

Several observers, notably Bendix and Bickel (15) and Mathews (16), have noted the spontaneous disappearance of sugar in alkaline solution. Although the quantity destroyed in this way is small, it might be sufficient in urines which had been rendered strongly alkaline by administration of sodium carbonate to account for a portion, at least, of the apparent retention. It was therefore necessary to assure ourselves that this possible error was at least not very large.

A specimen of urine was obtained by catheter from a completely depancreatized dog, its volume made up to 500 cc., and the entire quantity divided into two equal parts. The sugar in one portion was determined at once and found to be 1.49 gm. The second portion was made alkaline with sodium bicarbonate, placed in the incubator, and kept there at a temperature of 36°C. for 7 hours. At the end of this time the urine was found to contain 1.527 gm. of dextrose.

A second urine, faintly alkaline and containing 30 gm. of glucose for the 24 hours, was diluted to proper strength for titration by Benedict's sugar method. The titration for 25 cc. of the copper solution was 10.2 and 10.1 cc. After standing 6 hours in the incubator at 36° and 18 hours at room temperature the titration next day was 10.0 and 10.1 cc. Still another portion, made more strongly alkaline with sodium carbonate and kept beside the first control, gave a titration next day of 9.9 and 10.0 cc.

A similar control with sodium carbonate was performed by Elias and Kolb (4) in their work upon hunger diabetes.

Another factor that must be taken into account is the possible formation of complex condensation products of glucose, such as those found by Levene and Meyer (17) to be produced by the action of mixed extracts of pancreas and muscle upon sugar solutions in alkaline medium. To control this factor, a urine which had been rendered sugar-free by the administration of sodium carbonate to the animal from which it had been obtained, was boiled with a reflux condenser for 1 hour after acidifying with concentrated hydrochloric acid; it was then made alkaline with sodium carbonate and was tested for sugar with Benedict's qualitative sugar reagent, with negative results.

The Importance of Body Temperature.

Opinion is divided as to the influence which infection may have on the course of the diabetes produced by total pancreatectomy. Allen (18) reports many instances of peritonitis following partial pancreatectomy in which the infection appeared to be without specific influence on the course of the disease. Our own experience with partial pancreatectomy is very limited as yet, but we have seen instances of rapid death with high fever (40° and upward) following the total operation in which there seemed to be no other cause for the failure of glycosuria, and we have gained the impression that a serious infection with rise of body temperature is of the greatest moment. Accordingly, we have from the start kept a daily record of body temperature and are satisfied that we have spared ourselves much time and trouble by avoiding the use of animals with a high temperature.

A. The Administration of Alkali by Mouth.

It seemed strange, in view of the marked effect of alkali on the elimination of glucose by the depancreatized dog, that such effects had not long ago been observed after the administration of alkali to diabetic patients. Practically every diabetic patient in routine treatment has received alkali by mouth. It is commonly given in the form of sodium bicarbonate. Only in the face of threatening coma is the more strongly alkaline normal salt usually given or any alkali administered by vein. Patients of intelligence vouch for a marked improvement in their subjective feelings from the use of sodium bicarbonate and some claim the power to discern its need by an acid taste in the mouth or by a general feeling of depression. Why, then, has not some improvement in the glycosuria itself been observed? It is our belief that much of the benefit ascribed to the "oat cure" or other specific carbohydrate "cures" is really to be credited to the large amounts of sodium bicarbonate given at the same time. Weiland (19) says: "Wir haben zwar in allen Fällen dieser Art vom Hafer Gebrauch gemacht, ihn aber stets mit ausgiebiger Alkalizufuhr . . . . so dass eine eindeutige Haferwirkung nicht zu erkennen war. Viel grössere Bedeutung kommt hier der Zufuhr von Alkali zu." Possibly, oats and $NaHCO_3$ together supply the proper ratio of kations. In many of Weiland's cases the urine became sugar-free while large doses of alkali were being given. This has been seen by many other observers, but Weiland is the first, as far as we are aware, to recognize the alkali as a *possible* factor in diminishing the glycosuria. Recently, Underhill has reported a case that became sugar-free after the daily ingestion of $NaHCO_3$ in Vichy water had reached 120 gm. and was then kept in this state by smaller doses diminishing to 40 or 50 gm. (27).

As far as we can discover, nobody has attempted treatment of the dog made diabetic by pancreatectomy with alkali, after the manner of its use with patients. The question naturally arises whether the dog reacts differently in this respect from the human subject.

The experiments reported below show that in many instances there may be little or no effect from bicarbonate.

TABLE II.

Contrast between per Os and Intravenous Administration of Sodium Carbonate. Dog 3. Weight 7.95 Kg. Pancreatectomy July 18, 1914, 10 a.m.

Date.	Time.	Total G.	Total N.	G per hr.	N per hr.	G:N.	Blood sugar.	Sp. gr.	Temperature.	Remarks.
1914									°*C.*	
July 21	2.20 p.m.	2.55	0.952			2.73				
	2.30 "						0.292	1.068	38.3	17.89 gm. glucose given at 2.40 p.m.
" 22	10.15 a.m.	35.26	5.848	0.80	0.265	3.05				17.73 gm. recovered.
	11.00 "									5 gm. Na_2CO_3 by stomach tube.
	4.45 p.m.	7.05	2.00	1.08	0.307	3.52				
	4.50 "						0.198	1.078	38.2	
" 23	10.38 a.m.	11.244	5.92			1.89				
	2.08 p.m.						0.288	1.059		
	2.10–2.35 p.m.									Infusion 200 cc. distilled water + 1.5 gm. Na_2CO_3.
	5.45 p.m.	1.93	1.23	0.48	0.310	1.56				
	6.12 "						0.292	1.060		
" 24	Autopsy.	No pancreatic tissue found.								

Dog 3, whose pancreas was removed on July 18, 1914, at 10 a.m., had on July 21 a perfect Minkowski ratio and eliminated between 2.30 p.m. of this day and 10.15 a.m. the next day 17.73 gm. out of 17.89 gm. of glucose administered by mouth. The intolerance for carbohydrate was therefore complete. At 11 a.m. of the 22nd 5 gm. of sodium carbonate were given by stomach tube and at 4.45 p.m. urine obtained by catheter showed an increase

in the hourly elimination of both nitrogen and glucose, but the increase in the glucose was greater than that of the nitrogen, the result being, therefore, a higher G: N ratio. The blood sugar fell coincidentally with the rise in glucose excretion. At 10.38 a.m. on the 23rd the G: N was 1.89. The hourly elimination could not be computed. In the afternoon between 2.10 and 2.35, an infusion of 1.5 gm. of sodium carbonate in 200 cc. of distilled water was given. The G: N ratio fell to 1.56, the blood sugar and specific gravity changing only slightly. The normal salt, with its higher alkalinity, produced a prompt fall in the elimination of glucose when given by vein, but the effect was much delayed when given by mouth.

TABLE III.

Bicarbonate of Sodium and Potassium. Dog 8. Weight 8 Kg. Pancreatectomy August 26, 1914.

Date.	Time.	Total G.	Total N.	G per hr.	N per hr.	G: N.	Temperature.	Remarks.
1914							°*C.*	
Sept. 1	4 p.m.							10 gm. NaHCO$_3$. in 400 cc. water; all retained.
	6 "							100 gm. meat.
" 2	Cage urine.	4.84	1.764			2.74	38.5	
	Catheter 12 m.	5.85	2.548			2.29*	38.9	
	" 4 p.m.	1.10	0.728			1.51		500 cc. 1 per cent KHCO$_3$ at 4.10. 100 gm. meat at 5.30.
" 3	Cage urine.	6.33	1.932			3.27		
	Catheter 1 p.m.	5.18	2.478			2.09**		
	" 6 "							20 gm. NaHCO$_3$ up to 4 p.m. 200 gm. meat at 6 p.m.
	" 8 "	1.527	0.469	0.763	0.234	3.25**		
" 4	1 a.m.	7.780	4.51	0.518	0.300	1.72**	38.9	
	Catheter 11 "							
	" 1 p.m.	6.501	0.392	0.250	0.196	1.27*	39.1	
	Date of death lost. No pancreatic tissue found.							

* Urine acid.

** Urine alkaline.

Dog 8, depancreatized on Aug. 26, 1914, received on Sept. 1 at 4 p.m. 10 gm. of sodium bicarbonate in 400 cc. of water. At 6 p.m. 100 gm. of meat were eaten. The next morning the cage urine showed a G: N ratio of 2.74. At 12 m. urine from the bladder contained less glucose in proportion to the nitrogen, and the urine was acid, showing that the alkali had by this time been entirely eliminated. At 4 p.m., without further treatment, the ratio had fallen still further. Bicarbonate of potassium was then given in 1 per cent solution and the same quantity of meat was eaten. The next morning the ratio of glucose to nitrogen excreted in the cage urine was 3.27, while at 1 p.m. it had returned again nearly to the level it had shown at a corresponding time the day before when bicarbonate of sodium had been given. In brief, the two salts had produced nearly identical (if any) results. As will be shown in a later paper, the G: N of the depancreatized dog varies with the time after feeding, being above the Minkowski ratio in the early hours and below it in the later hours, but making an average for the 24 hours very close to the average given by Minkowski as characteristic for the meat-fed dog. Reilly, Nolan, and Lusk (20) have shown that the same is true, though the difference is much less striking, in the phlorhizinized dog; *i.e.*, the glucose derivable from protein is excreted more rapidly than the nitrogen. This is doubtless the meaning of the downward tendency of the ratio seen here after each feeding. Possibly, there was at the same time some delay in the gastric digestion caused by the presence of the alkali. On the evening of Sept. 3 the experiment with sodium bicarbonate was repeated, using, however, double the amount of alkali, and 2 hours later, double the amount of meat. At 2 and 7 hours later, first a high and then a low G: N was again found. At 1 p.m. the urine was acid, showing that the alkali had all escaped; but the ratio was still lower. At first sight, these observations appear to prove that the bicarbonates have a delayed effect in bringing the ratio down. There is reason, however, as we have seen, to believe that the ratio would have been somewhat lower at this time if the alkali had not been given.

Moreover, as the following experiment proves, bicarbonate administered to a fasting depancreatized dog may have the effect of causing sugar to reappear in the urine after it had entirely vanished.

TABLE IV.

*The Influence of Bicarbonate on a Fasting Depancreatized Dog. Dog 7. Weight 4 Kg. Pancreatectomy August 19, 1914. Fasting since August 15.**

Date.	Time.	Total G.	Total N.	G:N.	Temperature.	Reaction.	Remarks.
1914					°C.		
Aug. 21	Catheter 1.20–4.20 p.m.	0.0	0.525		38.7	Alkaline.	Refused all food.
	4.30 "						350 cc. 2 per cent $NaHCO_3$ by stomach tube.
	Specimen 6.30 "	0.695	0.266	2.61	38.7		Refused all food.
	" 7.05 "	0.631	0.283	2.23			
" 22	" 9.00 a.m.	1.238	0.794	1.56			
	Voided 10.00 "	0.185	0.595	0.3			Refused food.
	Catheter 11.30 "	0.0	0.192				
	12.00 m.						4 gm. $NaHCO_3$ in 300 cc. water by stomach tube.
	Voided 1.30 p.m.	0.185					
" 23		0.0					Refused food.
" 24	11.30 a.m.	0.0			39.3		" "
	3.15 p.m.						17.89 gm. glucose given in 300 cc. water; none vomited.
" 25	3.10 "	13.684			38.7		4.206 gm. retained.
" 28	Respiratory experiment. R. Q. after 20 gm. glucose, 0.80 and 0.79						
Sept. 2	Dog killed. No pancreatic tissue found. Neighboring tissues sectioned; no pancreatic tissue. Urine had been free of sugar to end.						

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* Pancreas was sclerotic and brittle; very easily removed.

Dog 7 had been in the laboratory for 4 days previous to operation and had refused all food during this time. The pancreas at operation was found to be quite sclerotic and brittle but was easily removed. 2 days afterward the urine, to our surprise, was found at 4.30 p.m. to be sugar-free. The dog meantime had eaten nothing. At 4.30, 350 cc. of 2 per cent solution of sodium bicarbonate were given by stomach tube and all was retained.

At 6.30, a G: N ratio nearly approaching the typical for meat-fed depancreatized dogs was obtained and the sugar continued in the urine until next day at 10 a.m. When, at 11.30, the urine was again found sugar-free, more bicarbonate was given and once more a small amount of sugar came out. The next 2 days, without any treatment, the urine continued sugar-free and on the 24th 20 gm. of commercial glucose, containing 17.89 gm. of actual glucose, by titration, were given at 3.15 p.m. 24 hours later 13.68 gm. had been excreted. This indicated a moderate tolerance, and judging from the results of the respiration experiment performed on the 28th, it is possible that some of the sugar was burned. *Autopsy revealed no pancreatic tissue although all fragments of tissue suspected of containing pancreas were sectioned.* The dog also was not pregnant unless in the very early stages when gross examination of the uterus would reveal nothing.

This interesting case will be alluded to in the third paper but is mentioned here for the purpose of showing that sodium bicarbonate, with its very slight alkalinity, may not only produce no reduction in the excretion of sugar, as seen in the last experiment, but may even call out sugar otherwise absent from the urine.

Dog 5, deprived of the pancreas on July 29, 1914, showed, 2 days later, a G: N ratio indicating a nearly total diabetes. By the administration of 7.5 gm. of the normal anhydrous salt in 1 per cent solution the urine was rendered sugar-free and this continued for 4 days under continued treatment with the alkali, notwithstanding the ingestion of 200 gm. of meat on one of these days. The blood sugar fell at first, then returned to 0.15 per cent, at which point it remained when last examined. This is a rather low percentage of blood sugar for total diabetes and, in order to make certain that the diabetes was total, it was necessary to make use of the respiratory quotient. Two periods on Aug. 4 were sufficient to establish the fact that there was no capacity on the part of the organism, even with the aid of the alkali administered by mouth, to oxidize glucose. After administration of 20 gm. of the sugar, with more carbonate, there was a slight rise in the R. Q. in the 2nd and 3rd hours, but it had completely subsided in the 4th. Many such experiments have been done in this same manner. The R. Q. and heat production have been

Dog 5. Weight 7.7 Kg. Pancreatectomy July 29, 1914, 2 p.m.

Date.	Time.	Temperature.	Glucose per hr.	Nitrogen per hr.	G : N.	CO_2	O_2	R. Q.	Blood sugar.	Sp. gr.	Remarks.
1914		°*C.*				*liters*	*liters*		*per cent*		
July 31	2.18		0.445	0.189	2.34				0.164	1.058	250 cc. 1 per cent Na_2CO_3
	5.10		0.383	0.219	1.74						500 " 1 " " "
Aug. 1		38.1	0.0						0.118	1.055	500 " 1 " " "
" 2			0.0								
" 3			0.0								200 gm. meat + 0.5 per cent Na_2CO_3.
" 4	10.30	38.2	0.0						0.153	1.060	300 cc. 1 per cent Na_2CO_3 by stomach tube. Vomited.
	Respiratory experiment.										
	12.15–1.15					2.916	4.439	0.66			300 cc. 0.5 per cent Na_2CO_3 subcutaneously.
	3.09–4.09					2.932	4.259	0.69			
	4.30	38.4	0.0	0.120					0.150	1.060	400 cc. 0.3 per cent Na_2CO_3 subcutaneously. 20 gm. glucose *per os*.
	Respiratory experiment.										
	5.27–6.27					2.926	4.283	0.68			
	6.27–7.27					3.083	4.319	0.71			
	7.27–8.27					2.789	3.863	0.72			
	8.27–9.27					2.919	4.260	0.69			
" 5	Dog dead.	No pancreatic tissue found.									

determined after the administration of carbonate alone and of carbonate with sugar several days after total pancreatectomy, but never have we seen a R. Q. higher than 0.73 *following the administration by mouth* of sodium carbonate with totally depancreatized dogs. As we shall see, the result is different after partial pancreatectomy.

B. The Administration of Alkali by Vein.

Our first positive result was obtained with Ringer's solution made alkaline to the extent of about 1 per cent with sodium carbonate.[1] Returning to this procedure, we have been able to reproduce the first result a number of times. The only failures were traceable to the use of bichloride of mercury in too concentrated a solution as a hand wash at operation. The following experiments are typical of the effects on urinary and blood sugar.

TABLE VI.

Dog 31. Pancreatectomy February 8.

Date.	Weight.	Urine.	Temperature.	Glucose.	N.	G: N.	Blood sugar.	Remarks.
1916	*kg.*		*°C.*				*per cent*	
Feb. 9	13.20	Catheter.	38.6	4.72	1.18	4.00		
		Cage.		17.73	6.02	2.94		
" 10	12.90	Catheter 9.40	37.6	0.50	0.18	2.78		
		" 12.40		2.18	0.364	5.9	0.40	1.00-1.15, 200 cc. Locke's solution + 1.5 gm. Na_2CO_3 by vein.
		" 3.40	40.2	1.49	0.49	3.04	0.42	
		" 7.00		0.00	0.196	0.0	0.38	
		" 10.00		0.00		0.0		

Dog 31. 2 days after operation 200 cc. of Locke's modification of Ringer's solution (containing, however, no glucose) were infused into the jugular vein. The infusion was stopped at 1.15

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[1] The addition of carbonate to Ringer's solution naturally precipitated the calcium, but this does not interfere with its use intravenously.

p.m., and at 3.40—2 hours and 25 minutes later—the sugar had completely disappeared from the urine. It was still absent at 10 p.m.

Three points are of special interest in this experiment: First, the high G: N ratio found at 12.40 p.m. The dog had exhibited the typical Minkowski ratio at 9.40 a.m. and, without having been fed, developed within 3 hours a ratio more than twice as high. The occurrence of high ratios at a corresponding period has been noted several times in the course of the work. An explanation of this peculiar behavior will be given in due time. At the moment it is of interest as showing how differently the depancreatized dog may behave from the dog kept uniformly intoxicated with phlorhizin (21). The second point of special interest is the decrease in nitrogen elimination coincidentally with the cessation of sugar elimination. The nitrogen output per hour falls to about half what it was before the infusion, but follows immediately upon a somewhat heightened excretion. In fact, if the whole elimination for the 6 hours be computed to the hourly basis it is seen that there is but very slight change from the rate at which the kidney was putting forth nitrogen previous to infusion. The more rapid elimination at first is doubtless due to a washing out process brought about by the dilution of the blood, and the slower elimination later is, in all probability, merely the natural compensation of this. Without the preliminary washing out one would be inclined to ascribe the whole change, as regards both sugar and nitrogen, to an effect on the kidney. Instead of a sweeping out of sugar, however, we see a gradual fall followed by total disappearance. The third point of interest is the behavior of the blood sugar. Were the effect of the infusion wholly a renal effect, one would expect an increase in total blood sugar[2] as the urinary sugar fell off, and there is indeed some indication of this in the slightly augmented percentage at the 3 hour period. Two facts prevent a definite conclusion from this experiment: The specific gravity was not determined, hence one cannot say to what extent the blood was diluted at this time; and, second, there is always a possibility that sugar has been withdrawn from the circulation under the influence of the alkali. This factor, as far as it may be related to a form of carbohydrate which can be mobilized again by adrenalin, will be discussed in the second paper.

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[2] See Appendix to Table III of the second paper for an instance of augmented blood sugar caused by failure of the kidney to produce urine.

TABLE VII.

Dog 32. Weight 12.55 Kg. Pancreatectomy February 13.

Date.	Time.	Temperature.	Volume of urine.	G.	N.	G : N.	Blood sugar per 100 cc. blood.	Sp. gr.	Remarks.
1915		°*C.*	*cc.*				*gm.*		
Feb. 16	Catheter 9.40	38.6		1.60	0.50	3.20			
	" 11.40		32	2.31	0.67	3.44			
	" 1.40		36	2.55	0.71	3.59	0.25	1.056	1.40–2.07, infusion of 300 cc. Locke's solution containing 3 gm. Na_2CO_3.
	" 3.40	40.0	100	1.64	0.71	2.31	0.24	1.049	
	" 5.40	38.8	30	0.00	0.46	0.00	0.20	1.043	
	" 7.45		200*	1.67	0.55	3.04	0.19	1.042	

* Dog had a large drink of water at 6 p.m.

Dog 32. On the 3rd day after operation, the G: N ratio in an early morning specimen from the bladder was high enough to indicate total diabetes. Two control periods of urine were obtained and a sample of blood was taken just before the infusion was begun. Within 2 hours the sugar had disappeared from the urine. Remaining absent for a complete 2 hour period, it was present again at the next examination 2 hours later in the same quantity as at the end of the first 2 hours. It is difficult to interpret the behavior of the nitrogen. Taking the figures in the control periods as normal, there appears to be a considerable fall coincident with the disappearance of sugar; but if the figure found at the end of the first 2 hour period after infusion may be interpreted as a rise due to washing out of extractive nitrogen, as was seen in the previous experiment, then the amount recovered at the last examination may be regarded as a return to the normal level which prevailed early in the morning.

Turning to the blood sugar, it is evident that the fall in percentage is not so great as would be expected from the change in specific gravity. The first step from 1.056 to 1.049 would be brought about by the addition of water to the extent of 25 per cent of the blood volume; the second drop to 1.043, by the addition of 46 per cent of the blood volume. If no sugar were held back by the kidney, such a dilution would produce a change in percentage from 0.25 to 0.20 per cent and the next step (1.043) would find it at the level of 0.17 per cent. Since only 100 cc. of urine were obtained at the end of the first 2 hour period, it is probable that more than enough water had actually been retained to account for the change in gravity (allowing 600 gm. of blood for a 12 kg. dog).

To produce the gravity found at the next examination, however, would require the retention of 275 cc. of water. It is possible that fluid might be withdrawn from the tissues to produce this result.

Assuming 600 gm. of blood at the beginning, 0.25 per cent of sugar would amount to 1.5 gm. as the total amount in circulation. At the time when the sugar was found entirely absent from the urine, however, there was 0.2 per cent in the blood, and if we assume that at least 275 cc. of water had been added to the circulating fluid (to produce the observed change in gravity), there must have been all told about 1.75 gm. of sugar in circulation, or an increase of 0.25 gm. Since before the infusion sugar was being excreted at the rate of 1.2 gm. per hour, whereas after the infusion only 1.6 gm. were recovered in 4 hours, it is evident that at least 3.2 gm. had been diverted. Only a small part of it can have remained in the circulation.

C. The Influence of Hydrochloric Acid on the G: N Ratio.

In the work previously reported the effect of a single dose of 2 per cent hydrochloric acid by stomach on the glycosuria of the depancreatized dog was noted (22). The rate of glucose elimination was increased for as much as 4 hours, while the rate of nitrogen remained constant. The same may be seen in the following experiments.

Dog 22, bull terrier, female, about 9 months of age, weight 7.9 kg., was depancreatized on Jan. 5, 1915. This dog was used for the determination of the mineral alkalinity of blood (23) in connection with the respiratory metabolism for several days. A complete Minkowski ratio on a meat diet was obtained on the 3rd day after pancreatectomy (Jan. 8). On this day, however, the dog refused food and, after it was given, vomited the entire meal. On Jan. 9 the following observations were made.

TABLE VIII.

Time.	G.	N.	G : N.	G per hr.	N per hr.	Remarks.
	gm.	*gm.*		*gm.*	*gm.*	
10.15 a.m.	3.97	2.52	1.58			
12.15 p.m.						300 cc. 0.15 per cent HCl by stomach tube.
1.30 "	4.24	1.00	4.24	1.30	0.30	
4.45 "	1.61	0.99	1.63	0.49	0.30	

Dog 27, brindle bull, female, probably about 8 months of age, weight 11.8 kg., was depancreatized on Jan. 29, 1915. On the morning of the 30th, without having eaten any food since operation, the dog showed a G: N ratio of 2.50. On the morning of Feb. 1 the ratio in the cage urine was only 1.78, but the bladder urine at 2.20 p.m. gave a ratio of 2.84. At 2.35, 100 cc. of 0.3 per cent hydrochloric acid were given subcutaneously. The results follow.

TABLE IX.

Date.	Time.	G.	N.	G : N.	G per hr.	N per hr.	Remarks.
1916		*gm.*	*gm.*		*gm.*	*gm.*	
Feb. 1	10 a.m.	23.53	13.25	1.78			
	2.20 p.m.	5.17	1.82	2.84	1.19	0.42	
	2.35 "						100 cc. 0.3 per cent HCl subcutaneously.
	5.20 "	5.84	1.62	3.60	1.94	0.54	
" 2	10.10 a.m.	25.00	8.77	2.83	1.47	0.51	

The results of these two experiments confirm in every particular those previously reported. With the nitrogen remaining constant, the sugar output per hour is increased in one instance about threefold. In the other, the nitrogen level is not so constant—a rise of approximately 30 per cent can be made out—but the glucose rises about 80 per cent in the first 3 hours and evidently continued high for some time thereafter.

Mention should be made at this point of the extreme toxicity of hydrochloric acid for the depancreatized dog. Eppinger (24) has shown that a dog of 6.5 kg. after removal of the pancreas succumbed to a dose of 2.7 gm. of hydrochloric acid, or about 0.4 gm. per kilo, and another of 7.6 kg. was brought to the point of impending death (when the dog was bled for the purposes of blood analysis) by a dose somewhat less than this. On the other hand, a normal dog (25) on a meat diet will withstand a dose of 0.9 gm. of hydrochloric acid per kilo and will neutralize 80 per cent of the acid with ammonia.[3] Confirmation of these observations has been seen in the course of this work.

Dog 25, a young female, weighing 7.4 kg., was depancreatized on Jan. 23, 1915. On the 25th she received 0.6 gm. of hydrochloric acid in 300 cc. of water by stomach tube. Two-thirds of this was vomited a half hour later. The same evening the dog was given a meal of meat and lard but it was vomited during the night. On the 26th at 1 p.m. 0.5 gm. of hydrochloric acid was given in 25 cc. of water subcutaneously. The next day the dog vomited excessively all the morning. At 12.50 p.m. 0.3 gm. more of hydrochloric acid was given in 100 cc. of water intraperitoneally. The dog died in coma at 6 p.m. The total amount of acid given was 1.4 gm., or altogether less than 0.2 gm. per kilo.

SUMMARY.

1. Sodium bicarbonate and potassium bicarbonate administered by stomach tube may be without immediate effect on the glycosuria and the hyperglycemia of the depancreatized dog.
2. A bicarbonate given by mouth to a fasting depancreatized dog may even cause the reappearance of glucose in the urine after it had been "starved out."
3. The normal anhydrous salt of sodium, Na_2CO_3, may, on the contrary, reduce the sugar in the urine materially when given

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[3] Schryver (26) has emphasized the importance of ammonia derivable from the products of tryptic digestion as a protection to the liver against the autolytic effects of acids.

by mouth, and when given by vein invariably does so, especially when added to Ringer's or Locke's solution to the amount of about 1 per cent.

4. The blood sugar does not undergo a compensating increase in percentage even when the dilution of the blood is accounted for.

5. Dilute HCl given by mouth or subcutaneously to the depancreatized dog has just the opposite effect of alkali—increasing the sugar in the urine without affecting materially the nitrogen elimination—and without causing any effect on the blood.

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PANCREATIC DIABETES IN THE DOG.

II. IS THE GLUCOSE RETAINED WHEN SODIUM CARBONATE I ADMINISTERED TO DEPANCREATIZED DOGS DEPOSITED AS GLYCOGEN?

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Recent work upon the problems of glycogenesis and glycogenolysis points clearly to the fact that sodium carbonate exerts an inhibitory effect upon those processes which cause a liberation of glycogen from its hypothetical intracellular combination and which bring about a cleavage of the glycogen molecule into glucose. Pavy and Bywaters (1) reported that the injection of acids into the portal vein, ante mortem, increases postmortem glycogenolysis, whereas the injection of sodium carbonate has the opposite effect. As early as 1882 Einhorn, working in Ehrlich's laboratory, showed that, while winter frogs neither lose glycogen from their livers nor form new glycogen when immersed in a plain sugar solution, they do lose their glycogen if the sugar solution is acidulated (2). On the other hand, when such frogs are immersed in a dextrose-sodium carbonate solution, a demonstrable formation of new glycogen from available glucose takes place without the occurrence of glycogenolysis. A diminution in glycogenolysis and even an actual glycogenesis has been observed by Elias (3) in turtle livers perfused with Ringer's solution containing sodium carbonate. Furthermore, Elias and Kolb were able to bring about a considerable loss of glycogen from the livers both of rabbits and dogs by the administration of large doses of 0.1 N hydrochloric acid (4). Macleod and Pearce (5) also have demonstrated the extreme sensitiveness of the glycogenolytic process to the percentage of CO_2 (hydrogen ion concentration) in the blood passing through the liver.

Thus the antagonism of acids and alkalies in accelerating or retarding the processes of glycogenolysis and glycogenesis seems clearly established for normal animals, and one cannot help thinking that the action of sodium carbonate in decreasing the intensity of the glycosuria, as reported in the preceding paper (6), may be due to the fact that it favors the retention of preformed glycogen and brings about a formation of new glycogen from available glucose.

Methods of Experimentation.

Two methods were used in an attempt to demonstrate a retention of glucose in the form of glycogen. First, by direct determination of the glycogen contained in the liver and muscles of a depancreatized dog in which a retention of glucose had been brought about by the administration of sodium carbonate; second, by a method suggested to us by Dr. R. T. Woodyatt, the steps of which were as follows: A completely depancreatized dog was deglycogenized by the repeated subcutaneous injection of small doses of adrenalin, as described by Sansum and Woodyatt (7). The animal was then fed on some glucogenetic material, usually lean meat. Sodium carbonate was administered in repeated doses, either *per os* or intravenously in 1 per cent solution, and after a considerable quantity of glucose had been retained, was again deglycogenized by repeated injections of adrenalin. The elimination of extra sugar was to be taken as proof that the sugar which failed to appear in the urine during the administration of the carbonate had been retained as glycogen. The detailed description of these experiments follows.

Experiment 1 (*Table I*).—The dog was depancreatized in the two-stage operation of Minkowski, but following the rapid technique of Hédon. The urine was then collected in definite periods of about 24 hours each and as soon as the G: N ratio reached 2.62, approximately the typical Minkowski ratio, the experiment was begun. The dog was given 2 gm. of sodium carbonate in 1 per cent solution by stomach tube at 10 a.m. and a second dose at 1 p.m. The G: N on the following day fell to 1.90. The total amount of sugar retained was 6.86 gm., the glucose retained thus being the difference between the amount that theoretically should have been eliminated and the amount actually excreted. The carbonate was continued for the next 48 hours in 2 gm. doses twice a day. On the 4th day 300 gm. of lean beef heart were given; on the 5th day, 400 gm., and on the 6th, another 300 gm. The administration of carbonate was continued in doses varying from 2 to 5 gm. until a total of 51.18 gm. of glucose had been retained. 2 hours before the animal was anesthetized 50 gm. of glucose and 5 gm. of sodium carbonate were given by stomach tube, thus supplying a large quantity of glucose for glycogenesis and of sodium carbonate to inhibit any glycogenolysis that might be caused by the etherization in the normal animal (8). Care was taken to allow sufficient air during the etherization and the excitement stage was thereby reduced. 9 minutes elapsed from the time that the anesthetic was begun until the first specimen of liver was removed and placed in the alkali solution. Glycogen was determined by the method of Pflüger. After prolonged hydrolysis with dilute hydrochloric acid the fluid was concentrated to a small volume by slow evaporation on the water bath, neutralized after cooling, and the sugar content determined by Benedict's titration method. As shown in the protocol of this experiment at the bottom of Table I, only amounts of sugar too small for quantitative estimation by Benedict's method could be demonstrated in the fluid after hydrolysis.

Experiment 2 (Table II).—This is reported in corroboration of the above except that in the second experiment no anesthetic was used in the final procedure.

TABLE I.

Experiment 1. Dog 6. Female. Weight 13.87 Kg. Depancreatized in Two Stages: First Stage, November 22, 1914; Second Stage, Transplant Removed December 4, 1914.

Date.	Time.	Total glucose.		Total nitrogen.	Glucose per hr.	Nitrogen per hr.	G : N	Reaction.	Temperature.	Remarks.
		Titration.	Polariscope.							
1914		*gm.*	*gm.*	*gm.*					°*C.*	
Dec. 6 " 7	3.30 p.m. to 3.10 p.m.	32.40	34.49	11.872	1.467	0.503	2.90	Alkaline.	38.4	Water.
" 10	9.30 a.m. to 7.30 p.m.	9.39	9.52	3.58	0.939	0.358	2.62	"	38.5	2 gm. anhydrous sodium carbonate in 200 cc. H_2O at 10 a.m. and 1 p.m. by stomach tube.
" 10 " 11	7.30 p.m. to 7.30 p.m.	15.30	15.71	8.03	0.637	0.334	1.90	"	38.4	Wound healing, clean. 2 gm. Na_2CO_3 in 200 cc. H_2O by stomach tube 7.40 p.m. Dec. 11. Retained 6.86 gm. glucose.
" 12	7.30 p.m. to 10 a.m.	11.53	12.85	5.51	0.623	0.297	2.09	"		At 10 a.m. 2 gm. Na_2CO_3 in 300 cc. water and at 2.10 p.m. by stomach tube. Retained 3.740 gm. glucose.
	7.30 p.m.	Urine contaminated with feces; discarded. Catheterized 7.30 p.m.								300 gm. lean beef heart given; all taken 7.40 p.m.; also 2 gm. Kahlbaum Na_2CO_3 in 300 cc. water. Feces analyzed for sugar; negative.
" 13	7.30 p.m. to 7.20 p.m.	30.34		17.86	1.264	0.744	1.69	Alkaline.	38.2	400 gm. lean beef heart at 7.30 p.m. + 4 gm. Kahlbaum Na_2CO_3. Retained 18.96 gm. glucose.

Dec. 14	7.20 p.m. to 7.15 p.m.	32.14		16.04	1.339	0.668	2.00	Alkaline.		Feces analyzed for sugar; negative. 5 gm. Kahlbaum anhydrous Na_2CO_3 and 300 gm. lean beef heart at 7.30. Retained 12.14 gm. glucose.
" 15	1.20 p.m.	16.87		9.54	0.937	0.530	1.76	"	38.4	At 1.40 p.m. 50 gm. Merck's c.p. dextrose + 5 gm. Kahlbaum sodium carbonate (anhydrous) given by stomach tube. All taken and none vomited. Retained 9.48 gm. Dog weighed 9.5 kg.

Dec. 15. Anesthetic (ether) started at 3.43 p.m. No excitement stage; allowed sufficient air. 3.52 p.m. First specimen of liver removed and in alkali solution 75 gm. 3.55 p.m. Second specimen of liver removed and in alkali solution 60 gm. 4.07 p.m. Third specimen of muscle removed and in alkali solution 75 gm. Dog stopped breathing 3.54 p.m. Specimens all contained but a faint trace of sugar after hydrolysis. Total glucose retained 51.18 gm. One urine period lost (contamination). 2.76 used as control G : N.

TABLE II.

Experiment 2. Dog 10. Female Terrier. Weight 4.6 Kg. Depancreatized in Two Stages: First Stage, May 2, 1916; Second Stage, Transplant Removed May 8, 1916.

Date.	Time.	Glucose.	Nitrogen.	G : N	Blood sugar.	Weight.	Reaction.	Remarks.
1916		*gm.*	*gm.*		*per cent*	*kg.*		
May 8-9	3 p.m.-10 a.m.	13.09	3.14	4.17	0.245	4.6	Acid.	150 gm. lean meat.
" 9-10	10 a.m.-10 a.m.	25.25	8.01	3.15	0.214	4.4	"	
" 10	1 p.m.							35 gm. glucose in 200 cc. water. Some lost.
	5 "							180 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
	5.30 p.m.							150 gm. lean meat.
" 11	10 a.m.	25.23	3.75	6.72	0.346		Alkaline.	Dog bled from wound at 8 a.m. Some urine lost.
	2-2.15 p.m.							150 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
" 12	10 a.m.	42.18	3.95	10.7	0.301	4.0	"	44 gm. glucose *per os*. 14.76 gm. glucose retained.
	10.45-11 a.m.							Injected 200 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
	11.30 a.m.							44 gm. glucose *per os*.
	6 p.m.							Injected 150 cc. of 1 per cent Na_2CO_3 in Ringer's solution intravenously.
	6.35 p.m.							Dog killed suddenly.

Temperature was normal during the entire experiment, 38-39°.

Results of analyses for glycogen, May 12:

6.35 p.m. Specimen 1 (liver) ground and placed in 60 per cent KOH = 0.092 per cent.
6.36 " " 2 " " " " " 60 " " = 0.059 "
6.40 " " 3 (muscle) " " " " " 60 " " = None.

Experiment 3 (*Table III*).—The animal was completely depancreatized in two stages and the urine collected in definite periods of approximately 24 hours' duration, the urine, as seen in Table III, showing a G: N of 3.07 for the first 23 hours and 2.98 for the next 24 hours. Adrenalin in 1: 1,000 dilution was then administered subcutaneously every 3 hours in doses of 0.25 to 0.5 cc. until further administration of adrenalin failed to raise the G: N. This was taken as indicating that the animal was now completely deglycogenized (7). The adrenalin was then discontinued and sodium carbonate in 1 per cent solution was given by stomach tube in repeated doses of 2.5 to 5 gm.

The dog was fed on lean beef in quantities varying from 200 to 500 gm. daily in one or two feedings. Even before the meat feeding was begun the G: N dropped from 2.66 to 2.31 following the administration of sodium carbonate. The G: N continued to fall even during the period in which the meat was being fed. Thus a considerable amount of glucose that must have been derived from the ingested meat failed to appear in the urine.

The adrenalin injections were then resumed with the object of bringing about the elimination of the supposedly retained glucose, as extra sugar, which would have been the case if it were retained as glycogen. That no such extra glucose appeared even after the injection of 0.5 cc. of adrenalin at 3 hour intervals, we are inclined to interpret as conclusive proof that glucose retained under the influence of sodium carbonate does not exist as glycogen. For suggesting this method of procedure we are indebted to Dr. Woodyatt.

The negative result in Experiment 1 and in other experiments similarly performed led us to think that the sugar which was apparently retained might have been excreted through other channels. Therefore, the possibility of glucose being excreted by the saliva, stomach, and intestine was investigated.

TABLE III.

Experiment 3. Dog 8. Female Bull Pup. Weight 8.4 Kg. Pancreatectomy: First Stage, February 26, 1915; Second Stage, March 3, 1915.

Date.	Time.	Glucose.		Nitrogen.	G : N	Weight.	Acetone.		Remarks.
		Titration.	Polariscope.				Gunning.	Rothera.	
1915		*gm.*	*gm.*	*gm.*		*kg.*			
Mar. 3–4	2.15–1.15 p.m.	32.02	32.12	10.42	3.07*	8.4	+	–	
" 4–5	1.15–1.30 "	34.89	32.12	11.70	2.98*		+	++	Adrenalin. See note 1 below.
" 5–6	1.30–1.30 "	33.88	32.12	10.47	3.23*	8.4	+	++	
" 6–7	1.30–1.20 "	33.79	31.39	11.73	2.88*	8.76	+	++++	
" 7–8	1.20–1.35 "	31.03	30.65	11.63	2.66*	7.75	+	++	
" 8	2.00 "								250 cc. of 2 per cent Na_2CO_3 by tube; partly vomited.
	5.30 "								500 cc. of 1 per cent Na_2CO_3 by tube.
	6.00 "	2.51	2.57	1.11	2.31**				
" 9	1.35 "	26.66	26.60	11.09	2.40**	8.00	–	–	See note 2. Lean meat and carbonate.
" 9–10	1.35–1.35 "	26.26	26.16	13.86	1.89**	7.59	–	–	
" 10–11	1.35–1.35 "	29.61		12.85	2.30**	7.37	–	–	
" 11–12	1.35–1.25 "	19.64	20.24	7.64	2.57**	6.69	+	+	Adrenalin. See note 3.
" 12–13	1.25–1.30 "	21.97	18.33	7.62	2.88*	6.35	–	–	
" 13	5.00 "	31.59		15.12	2.08**		–	–	Fed 600 gm. lean meat and 6 gm. sugar-free extract of pancreas.

* Urine acid.

** Urine alkaline.

1. 0.3 cc. adrenalin injected subcutaneously every 3 hrs. from Mar. 5, 1915, at 4.30 p.m. to Mar. 8 at 10.30 a.m.

2. Administered 25 gm. of sodium carbonate in 2.5 gm. doses, and 1,050 gm. lean meat *per os* in divided amounts from Mar. 8 at 9 p.m. to Mar. 11 at 1.40 p.m.

3. Adrenalin injections resumed on Mar. 11 and repeated every 3 hrs. until Mar. 13 at 10 a.m.

4. Dog anesthetized at 1.50 p.m. on Mar. 14. Right Wharton's duct exposed, cannula inserted, and about 2 cc. of [illegible] obtained which was found to contain sugar by both the Benedict and Fehling tests.

Appendix to Table III.

Mar. 14. Final experiment on Dog 8.

1.35–1.47 p.m. Saliva collected (ether stimulation); free flow. Safranin test positive, Benedict test negative. Fehling's positive after standing over night.

1.50 p.m. Ether anesthesia begun. Trachea exposed, tracheal cannula inserted and tied in.

1.58–2.02 p.m. Left femoral artery and vein exposed and cannulas tied in.

2.03–2.15 p.m. Abdomen opened. Liver found shrunken but of normal color and consistency and adherent to duodenum. Abdominal cavity clean but intestines matted together in adhesions at site of removal of pancreas. No pancreatic remnants found. Adhesions broken up, tube inserted into stomach and tied into esophagus, cannulas tied into pyloric ends of stomach and duodenum and lower end of ileum.

2.30 p.m. Stomach and intestine washed with normal saline and washings discarded.

2.45 p.m. Duct of right submaxillary gland exposed, cannula inserted. Chorda tympani nerve exposed and stimulated electrically (indirect interrupted current) 3.15–3.35 p.m., and saliva collected. A few drops of this saliva (mucin-free) gave a voluminous precipitate both with Benedict's and Fehling's reagents.

3.25 p.m. Both stomach and intestine again irrigated, the former with 500 cc., the latter with 1,000 cc. of 0.9 per cent NaCl. Washings collected, acidified with acetic acid, heated to remove protein, concentrated to less than 100 cc., neutralized and rendered alkaline with sodium carbonate, and tested for sugar. None found.

3.21 p.m. Blood collected from left femoral artery for determination of specific gravity and blood sugar.

Specific gravity.... $\begin{cases} 1.060 \text{ Hammerschlag method, using} \\ 1.058 \text{ chloroform and benzene.} \end{cases}$

Sugar analysis. ......0.327 per cent.

3.30 p.m. Injection of 1 per cent Na_2CO_3 solution into left femoral vein begun. Rate 3 to 8 cc. Total amount injected 200 cc.

3.40 p.m. Catheterized, about 50 cc. of acid urine obtained.

5.25 p.m. Injection of carbonate finished.

5.20 p.m. Chorda tympani nerve again stimulated and second sample of saliva collected, which also gave both strong Benedict and Fehling tests. Not as strong as the first with the same amount of saliva and reagent.

5.26 p.m. Blood collected for specific gravity and blood sugar.

Specific gravity............................ $\begin{cases} 1.047 \\ 1.050 \end{cases}$

Blood sugar..................................0.378 per cent.

5.30 p.m. Stomach and intestine washed with normal salt solution as before. No sugar found either by Benedict's or Fehling's test.

5.40 p.m. Bladder catheterized, no urine obtained. At autopsy the bladder was found contracted and empty.

5.48 p.m. Dog killed.

Examination of Saliva.

The saliva of Dog 8 (Experiment 3), as well as that of the dog used in Experiment 5, was collected at least once each day, the quantity obtained varying from 5 to 20 cc. When the saliva did not flow spontaneously, a flow was brought about by ether fumes blown into the mouth. Mucin was removed by adding acetic acid to the mixed saliva and the filtrate tested by at least one of three tests, Benedict, Fehling, or safranin, or by all three.

At the end of Experiment 3 the animal was anesthetized, the duct of the right submaxillary gland exposed, a cannula inserted into it, and saliva obtained by stimulating the chorda tympani nerve electrically at definite intervals, as indicated in Table III.

In Experiment 5 the pancreas was removed in two stages at an interval of 4 days, and when the G:N ratio reached the level of 2.89 saliva was collected and tested for sugar, with negative results. Further work on this dog was rendered impossible by the development of complications. It will be noted that even when the elimination of glucose in the urine was maximal the saliva contained no sugar (Table IV), nor were we able to demonstrate any appreciable amount of sugar after feeding meat and carbonate.

Examination of Feces and Intestinal Secretions.

Two specimens of feces were collected during the latter part of the experiment reported in Table I. These were extracted with acidulated water; the acid extract, heated to coagulate the protein, was then filtered, and the filtrate concentrated to small volume; it was then cooled, neutralized, and examined for sugar, but none was found in either instance.

In Experiment 3, as shown in the protocol, the abdomen was opened, cannulas were tied into the pyloric end of the stomach and duodenum and also into the lower end of the ileum. The stomach and intestine were washed at definite intervals with 1 liter of warm salt solution both before and after the intravenous injection of a 1 per cent sodium carbonate solution. The blood was also collected before and after the administration of the carbonate, and the blood sugar and specific gravity were determined. The changes observed in the blood are due to failure of the kidney.

All the washings were acidified with acetic acid, heated to coagulate the protein, filtered, the filtrate was concentrated to 100 cc. or less, and then made up to 100 cc., and the sugar content determined by Benedict's method. Results were negative. (See appendix to Table III.)

TABLE IV.

Experiment 5. Dog 10. Female Bull Terrier. Pancreatectomy: First Stage, April 29, 1915; Second Stage, May 3, 1915.

Date.	Time.	Total glucose.		Total nitrogen.	Glucose per 24 hrs.	Nitrogen per 24 hrs.	G : N	Temperature.	Saliva.	Remarks.
		Titration.	Polariscope.							
1915		*gm.*	*gm.*	*gm.*				*°C.*		
May 3-4	1.45 p.m.									Water.
	4.30 "	34.37	35.09	11.87	30.72	10.63	2.89	38.2		
" 4-5	2.30 "	32.37	32.12	11.34	35.32	12.36	3.03		Safranin and Benedict tests negative.	

TABLE V.

Experiment 6. Dog 7. Female, Weight 9 Kg. Depancreatized, February 6, 1915.

Date.	Time.	Total glucose.		Total nitrogen.	G : N	Temperature.	Remarks.
		Titration.	Polariscope.				
1915		*gm.*	*gm.*	*gm.*		*°C.*	
Feb. 8	A.m. specimen.	15.87	15.76	5.18	3.04	39.5	300 gm. lean beef heart.
" 9	" urine.	37.20	35.71	12.54	2.96		Fed 200 gm. beef heart and 3 gm. extract of pancreas.
" 10							Urine lost. 0.25 cc. 1:1,000 adrenalin (Parke, Davis and Co.) subcutaneously for four doses.
	8 p.m.	21.64	20.24	4.53	4.77	40.5	
" 11	A.m. specimen.		2.08	1.23	1.69		
	1.15 p.m.		2.08	2.55	0.81	39.0	
" 12	1.15 "		5.95	5.01	1.18	39.8	4 p.m., fed 300 gm. lean meat. Vomited 150 gm.
" 13	2.00 "	17.26		6.19	2.78		

Appendix to Table V. Experiment 6.

2.10 p.m. Dog anesthetized. Left Wharton's duct exposed and cannula inserted. Chorda tympani nerve of same side also exposed.

2.40 p.m. Chorda tympani stimulated for 10 minutes and saliva collected. Contained trace of glucose (Benedict's test).

2.50 p.m. Cannulas tied into pyloric end of stomach and duodenum and lower end of ileum; also stomach tube inserted into stomach.

3.50–4.00 p.m. Stomach washed with 1,000 cc. of 0.9 per cent sodium chloride solution; 900 cc. recovered. No reduction found after removing proteins by acid coagulation and heat and concentrating to 100 cc.

4.00–4.10 p.m. Small intestine washed. About 900 cc. of fluid recovered. No reducing substance found after similar treatment.

4.15–4.50 p.m. 160 cc. of 16.36 per cent solution of c. p. glucose (Merck's anhydrous) injected into left femoral vein.

4.55–5.05 p.m. Second intestinal irrigation. After similar treatment as first stomach washing, the fluid reduced Benedict's solution. Amount too small for quantitative estimation by Benedict's method.

5.05–5.17 p.m. Second stomach washing—1,000 cc. of solution used. Only 300 cc. recovered. No reducing substances present.

5.20–5.45 p.m. 170 cc. of 1 per cent anhydrous sodium carbonate injected into left femoral vein.

5.40–5.50 p.m. Third stomach washing. No reducing substances detected in wash. All fluid recovered.

5.50–6.00 p.m. Third intestinal washing contained a quantity of reducing substance equal to 0.104 gm. of glucose. All fluid recovered.

5.15 p.m. Chorda tympani stimulated and saliva collected. Gave strongly positive test with Benedict's qualitative solution. Two drops of saliva gave positive test.

Autopsy.—No peritonitis. Sloughing at site of adrenalin injection. Lower part of ileum adherent to abdominal wound.

The first part of Experiment 6 is reported as a control showing that when lean meat is fed to a dog after repeated injections of adrenalin the G: N is approximately 3.00. Pancreatectomy was performed on Feb. 6, 1915. On Feb. 8, analysis of a specimen of urine obtained in the morning yielded a G: N of 3.04. The dog was fed 300 gm. of lean beef heart and 3 gm. of extract of pancreas which was sugar-free. 200 gm. of beef heart and 1 gm. of pancreatic extract were fed after removing the morning urine. The urine on analysis gave a G: N of 2.96. The urine of the 10th was lost. Four doses of adrenalin were injected subcutaneously at 3 hour intervals on the 10th, and the urine obtained at 8 p.m. on the same day showed a G: N of 4.77. Adrenalin injections were then discontinued and on the 11th and 12th three specimens of urine gave G: N ratios varying from 0.81 to 1.69. But after the administration of 300 gm. of lean meat, of which 150 gm. were vomited, the ratio again returned to 2.78, the typical Minkowski ratio.

The procedure in the second half of this experiment was similar to that adopted in Experiment 3. The stomach and intestinal contents were washed out before and after infusion of glucose into the vein, and again after infusion of 1 per cent sodium carbonate. Only in one instance was any reducing substance obtained, and this may have been due to failure of the kidney as in Experiment 3.

In Experiment 7 the procedure was similar to that in the third experiment and here again, although 25.58 gm. of glucose were retained following carbonate injections, none of it appeared in the urine as extra glucose during the second course of adrenalin injections.

TABLE VI.

Experiment 7. Dog 21. Depancreatized in Two Stages: 29.9 Gm. of Pancreas Removed February 29, 1916. Remaining 6.8 Gm. Removed March 3, 1916. Weight 14.16 Kg.

Date.	Time.	Total glucose.		Nitrogen.	G : N	Blood sugar, volume per cent.	Weight.	Reaction.	Remarks.
		Titration.	Polariscope.						
1916		*gm.*	*gm.*	*gm.*			*kg.*		
Mar. 14–15	10 a.m.-10 a.m.	27.565	27.50	8.97	3.07	0.340		Acid.	
" 16	10 a.m.	20.045	20.62	6.89	2.90	0.346	12.36	"	0.3 cc. adrenalin injected subcutaneously every 3 hrs. from 11 a.m., Mar. 16–2 p.m., Mar. 18.
" 17	10 a.m.	45.454	42.98	8.96	5.07	0.336	12.24	"	
" 18	4 p.m.	25.67	25.90	9.70	2.64	0.336		"	Dog very weak. Expelled black feces (blood).
	4.15–4.35 p.m.								250 cc. 1 per cent Na_2CO_3 in Ringer's solution intravenously. Distinct improvement in general condition at once.
	4.50 p.m.								50 gm. glucose offered. All taken at once.
" 19	4.00 "	60.09	57.56	10.20	5.88	0.377			13.15 gm. glucose retained.
	4.20–4.45 p.m.								350 cc. 1 per cent Na_2CO_3 in Ringer's solution intravenously followed by 40 gm. glucose *per os*.
" 20	4.10 p.m. 5.00 "	47.21	47.74	7.014	6.73	0.241	11.50	Alkaline.	12.43 gm. glucose retained. 0.3 cc. adrenalin injected subcutaneously every 2 hrs.
" 21	4.10 "	18.23	17.10	6.85	2.66			Acid.	Total amount of glucose retained 25.58 gm. Temperature fluctuated between 37.9 and 39.5°:

DISCUSSION.

Sansum and Woodyatt (7) observed the constant increase of the G:N varying from 3.7 to 6 in completely phlorhizinized dogs on administering ether, nitrous oxide, or other narcotics. Thus, in one experiment etherization for 10 minutes yielded 3.88 gm. of extra glucose. In a second experiment, 30 minutes of anesthesia brought about an elimination of 7.98 gm. of extra sugar; and in the third experiment the same dog eliminated 11.26 gm. of extra sugar during a 2 hour anesthesia. This extra sugar they showed to be derived from preformed glycogen. Hence, it may be contended that etherization favors the mobilization of glycogen, for Macleod and Pearce (9) have shown that in anesthetized dogs there is a rapid disappearance of glycogen from the liver. Nevertheless, it is difficult to imagine, in view of Sansum and Woodyatt's results, that more than a small fraction of the 51.18 gm. of retained glucose in our Experiment 1 could have been mobilized in the short period of 9 minutes which elapsed from the time the anesthesia was begun until the first specimen of liver was removed. It might also be contended that the sugar was retained as glycogen, deposited to a large extent in the heart rather than in the liver or in the muscle. Cruickshank (10) has shown that, while the percentage of glycogen in hearts of normal dogs may be as high as 0.5 per cent, in depancreatized dogs it may be increased up to 1 per cent. That these assumptions will not suffice to explain the results obtained by the present writers is evident from Experiment 3 in which the second series of injections of adrenalin failed to bring about an excretion of extra sugar. The glycogen content of the heart muscle, as reported in Experiment 2, agrees well with similar analyses reported in Cruickshank's paper.

Carlson and Ryan (11) have reported the finding of glucose in the saliva of normal cats, the quantity of sugar present depending upon the gland from which the saliva was obtained. The quantity was considerably increased by the administration of ether but was present in appreciable amounts even in docile, unanesthetized cats, so that sugar could be readily detected by the ordinary Fehling test. These writers also report the case of a man whose urine contained more than 3.5 per cent of sugar. His saliva gave a positive safranin test although phenylhydrazine and Fehling's tests were both negative. In the animals experimented on there seemed to be a proportionality between the concentration of the sugar in the blood and the amount of glucose in the saliva. In 1904 MacCallum (12) reported the observation that the intravenous infusion of a M/6 solution of sodium chloride in sufficient quantity produces a hyperglycemia in rabbits. When the blood sugar reaches a certain concentration glucose is excreted by the kidneys, and if the kidneys are cut off from the circulation the glucose is excreted by the intestine and stomach. In a subsequent experiment MacCallum found sugar being excreted into the intestinal fluid even though the kidneys were actively functionating. He speaks of an "intestinal diabetes" and a vicarious functioning of the intestine in the elimination of glucose when the renal function is defective.

Fisher and Moore (13) were unable to confirm MacCallum's observations. They showed that simple hyperglycemia, induced by the hypodermic injection of morphine, by diabetic puncture, or by the intravenous injection of glucose or sucrose, never leads to an elimination of sugar into the gastro-intestinal tract. But if a solution of sodium chloride is injected into the animals thus treated in quantity and concentration which of itself does not bring about the excretion of sugar into the intestinal tract, sugar appears in detectable quantities in the stomach and intestine. They therefore explain MacCallum's results as being due to the combined effect of morphine and sodium chloride and interpret their own results as being attributable to the influence of the sodium ion in increasing the permeability of the intestinal epithelium.

The most recent work on the excretion of dextrose into the stomach and small intestine is that of Kleiner (14) who found definitely measurable quantities of dextrose in the small intestines of normal rabbits, but the experimental procedure adopted by Kleiner, which involved anesthesia, operation, and asphyxia by clamping the trachea, would tend to produce a high degree of hyperglycemia. Kleiner found more sugar in the intestine after injecting as much as 7 gm. of glucose per kilo of body weight in 20 per cent solutions than when 4 gm. of glucose per kilo in 11.4 per cent solution were injected. Furthermore, while 0.5 per cent of injected dextrose appeared in the intestines when the kidneys were active and the larger quantity of glucose was given, 2.2 per cent appeared when the smaller amount was administered and the kidneys cut out of the circulation.

Both Experiments 3 and 6, of our own series, seem to show clearly that in depancreatized dogs but little reducing substance is excreted into the small intestine and none into the stomach. And while the amount eliminated by way of the small intestine may be somewhat increased by the injection of large amounts of glucose into the circulation, it is nevertheless so small as to exclude any vicarious functioning of the gastro-intestinal tract in the excretion of sugar, even when the glycemia is very high. Likewise, the quantities of glucose eliminated by our diabetic dogs by way of the saliva were so small, even when the hyperglycemia must have been very high as a result of the combined effects of removal of the pancreas and injections of adrenalin, as to have no bearing upon our problem. On the other hand, when the amount of sugar in the blood is still further increased as a result of anesthesia or the direct introduction of glucose into the circulation in massive doses, and particularly when renal function is depressed by the anesthetic or exposure, then considerable amounts of glucose may be excreted by the saliva.

It would seem that the anesthetic (ether) increases the permeability of the epithelium of the salivary glands to the sugar of the blood.

While it is realized that the number of experiments reported does not justify sweeping conclusions, nevertheless the following may be tentatively suggested. (1) Glucose retained as a result of the administration of sodium carbonate to depancreatized dogs is not held back as glycogen; and (2) the sugar is not excreted by way of the saliva, stomach, or small intestine. The question whether it is oxidized and other related questions will be treated in the later papers of this series.

In conclusion we wish to thank Professor J. T. McClintock for his cooperation in this research and also others who kindly gave us their assistance in the operative work.

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PANCREATIC DIABETES IN THE DOG.

III. THE INFLUENCE OF ALKALI ON THE RESPIRATORY METABOLISM AFTER TOTAL AND PARTIAL PANCREATECTOMY.

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The preceding papers have shown that alkali may cause a complete disappearance of sugar from the urine of an animal after pancreatectomy or may cause the retention of a considerable amount of ingested glucose without; however, leading to the formation of glycogen. The natural inference is either that oxidation of the sugar is favored or the inflow of sugar from the tissues to the circulation is retarded to a rate slower than that by which it makes its escape from the body by the kidneys. Either effect would be beneficial to an organism suffering from a diminished pancreatic function, and the subject merited careful study.

The investigation of the respiratory metabolism after administration of sodium carbonate, the salt which has given best results in the study of the glycosuria and hyperglycemia, is attended with peculiar difficulties. The sign of improved oxidation of glucose is a rise in the respiratory quotient. But if any carbon dioxide were to be given off by the salt itself, whether set free in the stomach by hydrochloric acid and vomited, or set free by neutralization in the blood with acid phosphate and eliminated through the breath, a rise in respiratory quotient of no moment for the real processes of metabolism would be obtained. The most careful controls had consequently to be made and the experimental work has consumed an immense amount of time.

Our conclusion after some 3 years' intermittent occupation with the problem is that in the totally depancreatized dog in good condition, *i.e.*, not moribund, the alkali alone is almost always without effect on the respiratory metabolism. An increase of a few points at most in the respiratory quotient may be obtained. With the partially operated animal, however, the result is striking and immediate. Experiments in sufficient number to illustrate these facts will be presented in detail. In the interest of brevity many negative results will be omitted entirely.

We have obtained some evidence, also, that pancreatic material (whether merely as an extract or in virtue of the presence of living cells we are not yet prepared to say) is, when administered with alkali, of distinct benefit to the diabetic organism. Because of the impossibility of longer continuing our work jointly we are obliged to leave this and some other points at an inconclusive stage.

The significant experiments on animals anatomically wholly depancreatized may be classified as follows: (A) negative effects; (B) experiments of doubtful import; (C) positively favorable effects. On partially depancreatized animals we have only two experiments but they are both positive; that is to say, both show conclusive evidence of improved oxidation of glucose. Believing that these results are of hopeful augury in the treatment of diabetes, we present them in full.

Criteria of Improved Oxidation.

Throughout this work we have relied not merely upon the respiratory quotient but, wherever possible, have calculated the heat production as well and when the after effect could be compared with a basal metabolism have taken note of the dynamic action of the ingested glucose (1).

The criterion of the completely diabetic state from pancreatectomy is a respiratory quotient of about 0.69. Lusk (2) has shown that the G: N ratio which Minkowski found to be typical of the depancreatized dog on a meat diet, namely, 2.8, should correspond to a respiratory quotient of 0.697. La Franca (3), Falta, Grote, and Staehelin (4), and the writers (5) have elsewhere reported quotients for totally depancreatized dogs varying from 0.67 to 0.71. Many quotients confirmatory of these observations will be found in the following pages. A discussion of the relationship between respiratory quotient and G:N ratio is reserved for a later paper. The experimental procedure has usually been the following. After establishment of the true diabetic respiratory quotient (and basal metabolism), alkali alone or alkali with glucose was administered, sometimes by stomach tube, sometimes by intravenous infusion, and the animal was returned to the respiration machine for 1 to 4 hours' observation. In a few instances, not here recorded, the time has been extended to 6 hours.

Experiments on Totally Depancreatized Dogs.

A. Negative Effects.

Dog 3, a young female bull terrier, weighing about 8 kg., was operated on July 18, 1914. 3 days later the G:N ratio on a meat diet was 2.79. The same day R. Q.'s of 0.71 were obtained before and after administration of 17.89 gm. of pure dextrose by stomach.[1] On the 22nd, 5 gm. of sodium carbonate in 250 cc. of water were given by stomach, and quotients of 0.70, 0.71, and 0.69, successively, were obtained in the 4 hours immediately following. On the 23rd, with the G: N ratio at 1.89, the following experiment was carried out.

TABLE I.

Dog 3. Weight 8 Kg. Pancreatectomy, July 18, 1914.

Date.	Weight.	Time.	Respiratory metabolism.			Remarks.
			CO_2	O_2	R. Q.	
1914	*kg.*	*p. m.*	*liters*	*liters*		2.10–2.30 1.5 gm. Na_2CO_3 (anhydrous) in 200 cc. distilled water infused by vein.
July 23	7.7	3.42–4.27	2.53	3.66	0.69	
		4.27–5.13	2.68	3.97	0.68	
						6.12–6.35 1.8 gm. Na_2CO_3 (anhydrous) in 100 cc. distilled water into femoral vein; 10 gm. glucose by stomach tube.
		7.18–8.08	2.76	3.82	0.72	
		8.03–8.49	2.85	4.17	0.68	

It is possible, judging from subsequent experience, that not enough glucose was given in this instance to insure penetration of the intestinal wall in sufficient amount to produce a pronounced rise in R. Q. The slight increase from 0.68 to 0.72, beginning within an hour after the infusion ceased, is not regarded as of great significance.

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[1] See Dog 3, Paper I of this series.

Dog 54, a female fox terrier, was depancreatized by the two stage operation. All the organ except a small portion of the uncinate process engrafted under the skin at the abdominal incision was removed on June 23, 1916. For a week the dog was fed the mixed diet employed by one of us in a series of papers on the pregnancy of the dog (6) and known in the papers of Professor Lusk as "the standard diet." On June 30 the graft was removed and the animal promptly became diabetic. Some preliminary observations on July 3 and 5 proved that the dog still had some capacity to oxidize glucose but on the 6th this capacity, as judged by the respiratory quotient, had wholly disappeared.

TABLE II.

Dog 54. Female Fox Terrier. Weight 7 kg. Pancreatectomy: First Stage, Graft under Skin, June 23, 1916; Second Stage, June 30, 1916. Mixed Diet between Operations.

Date.	Weight.	Temperature.	Time.	Respiratory metabolism.			Remarks.
				CO_2	O_2	R. Q	
1916	*kg.*	*°C.*		*liters*	*liters*		
July 6	5.7	38.2	10.46–11.46	2.07	2.90	0.71	Basal, *i.e.*, following food evening before.
			11.46–12.46	2.34	3.44	0.68	
							1 p.m. 30 gm. glucose in 200 cc. Ringer's solution + 0.05 N NaOH by stomach.
			1.49– 2.49	2.53	3.62	0.70	
			2.49– 4.05	3.71	5.11	0.71	
			4.05– 5.05	2.79	3.98	0.70	Dog urinated or vomited 2.05, after 2.50, and during third period.
" 8	5.75	38.0	10.17–11.24	2.34	3.20	0.71	Basal.
			11.24–12.19	2.22	3.03	0.71	12.40, 30 gm. glucose in 200 cc. Ringer's solution + 0.05 N NaOH by stomach.
			3.01– 4.00	2.57	3.71	0.69	
			4.00– 5.00	3.04	4.32	0.70	
			5.00– 5.58	2.94	4.13	0.71	Urinated during every period, but lapped it up at once.

TABLE II—*Concluded.*

Date.	Weight.	Temperature.	Time.	Respiratory metabolism.			Remarks.
				CO_2	O_2	R. Q.	
1916	*kg.*	*°C.*		*liters*	*liters*		
July 9							2.15 p.m. 200 cc. Ringer's solution + 0.05 N NaOH + pancreas extract by stomach.
" 10	5.8	38.4	10.47–12.04	2.66	3.91	0.68	Basal.
			12.04– 1.04	2.31	3.22	0.72	
							2.30 p.m. 200 cc. pancreas extract in Ringer's solution + 0.05 N NaOH by stomach tube.
			2.45– 4.00	2.66	3.79	0.70	
			4.00– 4.40	1.78	2.55	0.70	
" 11	5.9	38.2	11.45–12.15				150 cc. pancreas extract in Ringer's solution + 0.05 N NaOH intravenously.
							12.35 p.m. 30 gm. glucose by stomach tube.
			12.52– 1.52	2.62	3.74	0.70	
			1.52– 2.52	3.57	5.01	0.71	

We shall see later that a similar treatment with pancreatic substance in Ringer's solution, made alkaline to 0.05 N with pure sodium hydroxide, given with sugar gives in some instances a marked rise of quotient and of heat production. In the present instance, however, it failed completely. So far as is known, the experimental conditions were good except for the fact that the animal urinated frequently in the metabolism cage. The dog was always quiet at the termination of the periods and we have always found that the residual analyses for water and carbon dioxide in the air of the cage, with corrections for temperature and pressure variations which we employ, are reliable (7). Our only explanation for complete failure in this instance is that the latent time[2] within which the favorable effect of alkali may be expected varies considerably in different animals, and that the present case is one in which a much longer time than we allowed might be necessary.

B. Experiments of Doubtful Import.

Two of our experiments on the respiratory metabolism, while positive in character, are of doubtful import because of peculiar circumstances.

Dog 7 (Paper I, page 489), a black female, weighing about 4 kg., had refused all food for 4 days previous to operation and had continued to fast for 2 days, whereupon the urine was found to be free of sugar. On the 2nd day sodium bicarbonate had called out in 2 hours sugar in sufficient amount to make a G: N ratio of 2.61 (page 489). On the 8th day following pancreatectomy the dog had excreted in 24 hours only 13.68 gm. of glucose out of 17.89 gm. of pure dextrose (Merck) given by stomach tube and had yielded respiratory quotients indicating combustion of some carbohydrate. On the 28th, the 9th day, the experiment below was carried out.

TABLE III.

Dog 7. Weight 4 Kg. Pancreatectomy, August 19, 1914.

Date.	Time.	Respiratory metabolism.			Remarks.
		CO_2	O_2	R. Q.	
1914		*liters*	*liters*		
Aug. 28	10.30 a.m.				Given 20 gm. glucose (Merck) by stomach tube.
	12.10-1.10 p.m.	2.37	2.96	0.80	
	1.20-2.20	3.37	4.28	0.79	200 cc. 0.5 per cent Na_2CO_3 by vein, finished at 3.50 p.m.
					4.00 p.m. 20 gm. glucose (Merck) by stomach tube.
	5.04-5.54	2.45	2.87	0.86*	

* This was a perfect period, but the apparatus developed a leak in the one following and the experiment had to be discontinued.

The peculiar circumstance in connection with this animal was that the pancreas was found to be sclerotic and brittle. It shelled out of its sheath almost like cold fat. Whether, in view of this fact and the absence of anything resembling pancreatic cells in the surrounding tissues, the case should be classed as one demonstrating the benefit of fasting, we are uncertain. There was a positive reaction to the alkali and sugar but the case is atypical among our positive results with totally depancreatized animals. It behaved more like a partially operated dog (see below).

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[2] See Paper I of this series.

Dog 20, operated 10 days previous to the observations on the respiratory metabolism, had eaten well and had exhibited perfect Minkowski ratios. On Dec. 21, 1914, on a meat and lard diet, the G: N in the morning urine was 2.72. Sodium carbonate was given with the food on this day and the next morning the G: N had fallen to 1.62. On the 23rd, following meat and lard and more carbonate on the evening of the 22nd, the urine drawn by catheter at 9.55 was clear of sugar. The blood sugar had fallen to 0.11 per cent.

TABLE IV.

Dog 20. Weight 9.5 Kg. Pancreatectomy December 11, 1914.

Date.	Time.	Urine.				Respiratory metabolism.			Remarks.
		Source.	Total glucose.	Total nitrogen.	G : N	CO_2	O_2	R. Q.	
1914						*liters*	*liters*		
Dec. 21	9.45 a.m.	Catheter.	4.76	1.75	2.72				
	1.10 p.m.	"	3.33	0.88	3.79				
	6.00 "								300 gm. meat, 30 gm. lard, 15 gm. bone ash, 2 gm. Na_2CO_3.
" 22	10.05 a.m.	"	16.78	10.34	1.62				
	1.10 p.m.	"	0.0	1.19					
	1.15 "								Same diet. All eaten before 4.30. 2 gm. Na_2CO_3 extra at 5 p.m.
" 23	9.45 a.m.	Cage.	2.07	12.00	0.17				
	9.55	Catheter.	0.0						0.11 per cent sugar in blood.
	10.00–10.15	30 gm. glucose + 2 gm. Na_2CO_3 by vein.							
	11.30								0.40 "
	12.23– 1.23					2.746	3.051	0.90	
	1.23– 2.23					2.886	3.387	0.85	
	2.23– 3.23					2.625	3.072	0.85	
" 24	Autopsy: No pancreatic tissue.								

When 30 gm. of sugar and 2 gm. of sodium carbonate (anhydrous) were given by vein at 10 a.m. and the dog was placed in the respiration apparatus, quotients of 0.90, 0.85, and 0.85, successively, were obtained. The dog had eaten well up to the night before and seemed in good condition for the experiment but was found dead the next day, and we had no opportunity to establish further controls. It may be remarked here that the same glucose (Merck's dextrose), when aerated with a 1 per cent solution of sodium carbonate, gave off no carbon dioxide. Hence, the result is probably due to a true combustion, but a combustion which cannot of necessity be ascribed to the treatment. Probably every student of pancreatic diabetes in the dog has witnessed improvements, especially as regards the excretion of sugar, a day or two previous to the death of the animal. Minkowski (8) states that sugar, which at the height of the diabetes is excreted, will, at a stage when the animal is scarcely able to move, be held back, and that twice in his long experience he had seen sugar entirely disappear from the urine. Minkowski thought the change was due to a falling off in sugar production rather than the vicarious participation of some other organ.[3] Whatever explanation is finally found for the disappearance of sugar will probably cover the apparent improvement now ascertained, we believe, for the first time,[4] regarding the respiratory metabolism.

C. Positive Effects with Sodium Hydroxide and Pancreatic Extract.

Three instances of clear-cut beneficial effects on the respiratory metabolism of completely depancreatized dogs by the administration of sodium hydroxide in Ringer's solution may now be cited. In two of them pancreatic extract was given with the alkali.

Dog 45 exhibited on the 2nd day after operation an average R. Q. of 0.70 and the same 3 days later. Since the R. Q. seems to be a safer criterion than the G:N ratio, one feels no hesitation in saying that this dog, fed right along on meat diet, was actually an instance of total diabetes. Nevertheless, by administration of 175 cc. of 0.1 N sodium hydroxide in Ringer's solution, the R. Q. was raised some five points and

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[3] Minkowski (8), page 110.

[4] Several other instances of this could be cited from our own results.

TABLE V.

Dog 45. Young Female. Weight about 7.0 Kg. Pancreatectomy December 2, 1915.

Date.	Weight.	Temperature.	Time.	Respiratory metabolism.			CO_2 in venous blood.	Sugar in blood.
				CO_2	O_2	R. Q.		
1915	*kg.*	*°C.*		*liters*	*liters*		*per cent*	*per cent*
Dec. 3		38.2						
" 4	6.80	38.2	11.45- 1.03	4.290	5.944	0.72		
			1.03- 1.45	2.252	3.324	0.68		
" 7	6.35	38.0	10.26-11.26	2.593	3.773	0.69		
			11.26-12.26	2.832	3.946	0.72	52.17	0.29
			1.05- 1.20	Infusion by jugular vein of 175 cc. Ringer's solution + 0.1 N NaOH.				
			2.55- 3.55	3.067	4.019	0.76		
			3.55- 5.00	3.322	4.414	0.75	65.52	0.24

the total heat production, as judged by the oxygen intake, was also augmented. The larger percentage of CO_2 in the venous blood shows that the R. Q. would have been even higher if all had been eliminated. The dog was quiet throughout. No sugar was given, but there was abundance of it in the circulation and, if the dynamic effect and the rise in R. Q. together do not indicate combustion, then we have no criteria which can be relied upon.

Dog 51, by all criteria was completely diabetic on the 3rd day following pancreatectomy. Ringer's solution[5] plus 1 per cent sodium carbonate by vein did not affect the R. Q. on the 6th day although the G: N was distinctly lower 2 days later and a second dose did not reduce it further; in fact, there was a slight rise in G: N. 4 days after this—the 12th since operation—a dose of Ringer's solution, in which the pancreas of two dogs had been allowed to macerate under toluene for 2 months in the ice box, was made alkaline to the extent of 0.05 N by addition of pure sodium hydroxide and was given with 30 gm. of pure dextrose by stomach tube. Immediately preceding the experiment the R. Q. had been at the level of complete diabetes.[6] The effect on the R. Q. and on the heat production was seen within an hour. Some doubt was thrown on the results by vomit-

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[5] Adding sodium carbonate to Ringer's solution precipitates the calcium as carbonate, but this does not interfere with its administration intravenously, as the carbonate probably rapidly becomes bicarbonate by taking up more carbon dioxide.

[6] Murlin (7). The probable error on the respiratory quotient for two successive periods with the respiration incubator which was used in all this work is not more than ± 0.02.

TABLE VI.

Dog 51. Pancreatectomy May 17, 1916.

Date.	Weight.	Time.	Glucose per hr.	Nitrogen per hr.	G : N.	Respiratory metabolism.			Calories per hr.	Remarks.
						CO₂	O₂	R. Q.		
1916	*kg.*					*liters*	*liters*			
May 20	9.55	9.28 a.m.								Dog fed standard diet of meat and lard.
		9.59–10.59				4.56	6.69	0.68	30.85	
		10.59–11.59				4.45	6.14	0.71	28.30	
		12.10 p.m.	1.00	0.30	3.3					
" 23	9.00	10.00 a.m.								
		10.40–11.40				4.06	5.62	0.72	25.66	
		11.40–12.40				3.69	5.15	0.72	23.37	
		1.52 p.m.	0.66	0.30	2.2					
		2.45–3.00								Given 140 cc. 1 per cent Na_2CO_3 in Ringer's solution by jugular vein.
		3.22– 4.22				4.09	5.97	0.70		Dog urinated in cage and defecated.
" 25		9.35 a.m.			1.8					
		11.35 "	0.79	0.46	1.7					
		12.25–12.45								Injected 150 cc. Ringer's solution freshly made + 1 per cent Na_2CO_3 by femoral vein.
		2.35 p.m.	0.86	0.53	1.6					
		4.35 "	1.17	0.50	2.4					
" 29	7.90	9.53 a.m.			0.63					
		10.49–11.49				2.78	4.20	0.66	19.79	
		11.49–12.49				3.19	4.67	0.68	22.03	
		1.05 p.m.	0.36	0.25	1.5					
		1.25 "								Given 200 cc. Ringer's solution + 0.05 N NaOH + pancreas extract + 30 gm. glucose by stomach.
		2.28– 3.28				4.38	5.88	0.75		Dog vomited in cage.
		3.28– 4.28				4.36	5.58	0.78		"

TABLE VI—*Concluded.*

Date.	Weight.	Time.	Glucose per hr.	Nitrogen per hr.	G : N.	Respiratory metabolism.			Calories per hr.	Remarks.
						CO₂	O₂	R. Q.		
1916	*kg.*					*liters*	*liters*			
May 31	7.55	10.03 a.m.			1.8					
		10.10 "								Given 30 gm. glucose by stomach tube.
		12.14– 1.14				3.58	4.57	0.79	20.39	
		1.14– 2.14				3.57	4.61	0.77	20.39	
		2.32 p.m.		0.52						
		2.36 "								Given 30 gm. glucose in Ringer's solution + 0.05 N NaOH + pancreas extract by stomach tube.
		3.04– 4.04				3.74	4.76	0.79	21.30	
		4.04– 5.04				4.42	5.09	0.87	23.41	
June 1	7.35	9.05 a.m.								
		9.42–10.42				2.77	3.54	0.78		
		10.42–11.42				3.12	4.01	0.78		
		1.52 p.m.	0.20	0.13	1.5					
" 2	7.25	9.46 a.m.								
		10.27–11.27				2.85	3.65	0.78		
		11.27–12.27				3.21	4.42	0.72		
		12.45 p.m.								200 cc. Ringer's solution + 0.05 N NaOH + pancreas extract + 50 gm. glucose by stomach tube.
		1.55– 2.55				3.16	4.11	0.76		
		2.55– 3.55				3.55	4.80	0.72		
		3.55– 4.55				4.46	5.74	0.75		

ing in the respiration chamber and the experiment was repeated 2 days later. To our surprise, the R. Q. after sugar alone was even higher than before, but again the combination of alkali, pancreas extract, and sugar in Ringer's solution carried it to a higher level. This time the heat production could be accurately calculated because the urinary nitrogen was obtainable and showed again what was clearly indicated by the rise in oxygen absorption in the first instance; namely, a considerable dynamic effect from the increased combustion. Subsequent attempts to raise the quotient still further by giving more glucose did not succeed. It is worthy of note, however, that Dog 51 lived for 11 days following the last attempt and continued to eat a mixed diet for more than a week.

Several animals (*e.g.*, Dog 54) were sacrificed in the attempt to repeat this experiment on Dog 51 but with only partial success thus far. An increase of ten points in the quotient, with an accompanying dynamic action, in an animal known to be completely deprived of internal pancreatic function at a time when the deficiency was most serious, is the most hopeful sign we have encountered in our work that it may yet be possible artificially to restore this function not only to operated dogs but also to men suffering with diabetes mellitus. Consequently, we have spared no pains to repeat the experimental conditions under which the above results were obtained.

Dog 53 is the only other animal which has yielded profitable results. Here again the dog is known to have been completely diabetic at the start. The first administration of alkaline Ringer's solution containing pancreas extract and sugar raised the quotient very distinctly the second hour, and by a second trial the quotient was kept at a level indicating the combustion of protein with all the glucose it can yield. The third attempt (on June 29) resulted in only partial success as there was evidently a leak in the apparatus the first hour. The dog was fed a large amount of carbohydrate following this experiment, with the hope of filling up the glycogen reservoirs and of obtaining something approaching normal combustion the next day, but the next morning the dog was found dead from some unknown cause. The respiration apparatus was checked frequently by combustion of alcohol during this season of work and is known to have been in perfect condition.

Possible Rôle of Pancreatic Extract.

So far as known to the writers, nobody hitherto has prepared a pancreatic extract on the assumption that the alkali which the pancreas is capable of producing may be of some significance for its internal function as well as for its external enzymes. As will be seen from Paper IV of this series,[7] there is some ground for such an assumption; hence, it would seem that a pancreatic extract designed to replace the lost hormone should be contained in an alkaline medium. Allen (9) has emphasized the fact that pancreatic extracts cannot be expected to replace the natural

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[7] To be published later.

TABLE VII.

Dog 53. Pancreatectomy June 23, 1916.

Date.	Weight.	Time.	Respiratory metabolism.			Remarks.
			CO₂	O₂	R.Q.	
1916	*kg.*		*liters*	*liters*		
June 26	6.33	11.10–12.10	3.01	4.48	0.67	250 gm. lean beef for diet.
		12.10– 2.05	3.01	4.40	0.69	
		2.05– 3.05	2.44	3.33	0.73	
" 27	6.25	10.04–11.00	3.13	4.22	0.70	
		11.00–12.00	3.33	4.74	0.70	
		12.05				Given 200 cc. Ringer's solution + pancreas extract + 0.05 N NaOH + 30 gm. dextrose by stomach tube.
		1.14– 2.14	3.72	5.16	0.72	
		2.14– 3.14	3.70	4.45	0.83	
" 28		12.30				Given 200 cc. Ringer's solution + pancreas extract + 0.05 N NaOH + 30 gm. dextrose by stomach tube.
	6.40	1.07– 2.07	3.92	5.09	0.77	
		2.07– 3.07	3.72	4.69	0.79	
		3.07– 4.07	4.10	5.68	0.72	
" 29	5.90	10.00–11.00	3.05	4.11	0.74	
		11.00–12.00	2.76	3.59	0.77	
		12.45				Given 200 cc. Ringer's solution + pancreas extract + 0.05 N NaOH by stomach tube.
		3.35– 4.35	2.92	4.30	0.68?	Leak.
		4.35– 5.35	2.60	3.30	0.79	

product, which is essential to the combustion of carbohydrate, unless the pancreatic cells are preserved. Acting on this suggestion and emboldened by the announcement of Kleiner and Meltzer (10) that an "emulsion" of pancreatic material had appeared to afford some assistance in disposing of extra sugar injected into the circulation of diabetic animals, we prepared the extracts used in the preceding experiments by grinding fresh dog pancreas in Ringer's solution and placing the resulting suspen-

TABLE VIII.

Dog 37. Partial Pancreatectomy August 3, 1915, Leaving About 2 Gm.

Date.	Weight.	Nature of experiment.	Respiratory period.	Respiratory metabolism.			Calories per hr.	Remarks.
				CO_2	O_2	R. Q.		
1915	*kg.*			*liters*	*liters*			
Aug. 9	6.0	Basal.	2.34– 3.34	2.328	3.220	0.72	15.14	Fasting 24 hrs.
			3.34– 4.38	2.385	3.154	0.76	14.99	
" 11	5.7	"	1.02– 2.02	2.235	3.073	0.73	14.49	Standard diet Aug. 10 5 p.m.
			2.02– 3.02	2.317	2.682	0.86	13.08	
			3.02– 4.02	2.300	3.023	0.76	14.36	
" 20	5.7	"	3.22– 4.22	2.457	3.247	0.76	15.43	Standard diet Aug. 19 5 p.m.
			4.22– 5.22	2.344	3.016	0.78	14.41	
			Average	2.338	3.059	0.77	14.55	
" 23	5.55	After 50 gm. glucose at 3.45.	5.05– 6.05	2.463	2.920	0.84	14.16	
			6.05– 7.05	2.392	2.675	0.89	13.14	
			7.05– 8.05	3.003	3.281	0.92	16.24	Fasting previous 24 hrs.
" 27	6.1	After 50 gm. glucose at 10.15 a.m.	10.52–11.52	2.520	3.001	0.84	14.56	Standard diet 3 days.
			11.52–12.52	2.616	2.913	0.90	14.34	
			12.52– 1.52	2.813	3.210	0.88	15.72	
			Average	2.635	3.000	0.88	14.69	
" 28	6.1	After 50 gm. glucose and 3 gm. Na_2CO_3 at 9.23.	11.13–12.13	2.963	3.089	0.96	15.44	
			12.13– 1.13	3.581	4.160	0.86		Dog vomited at 12.22.
			1.13– 2.13	2.968	2.937	1.01	14.83	

Aug. 31	5.85	Basal.	10–57–11.57	2.293	3.151	0.73	14.85	Standard diet Aug. 30 5 p.m.
			11.57–12.57	1.997	2.480	0.80	12.81	
			12.57– 1.57	2.361	3.168	0.74	15.02	
			Average	2.217	2.933	0.76	14.23	
" 31	5.85	After 3 gm. Na_2CO_3 alone at 2.45.	3.56– 4.56	1.921	2.568	0.75	12.17	
			4.56– 5.56	2.191	2.835	0.77	13.51	
			Average	2.056	2.701	0.76	12.84	
Sept. 4	6.4	50 gm. glucose + 3 gm. Na_2CO_3 at 9.45.	10.35–11.35	2.506	3.051	0.82	14.73	Standard diet Sept. 3 5 p.m.
			11.35–12.35	2.739	2.820	0.97	14.13	
			12.35– 1.35	3.004	3.380	0.89	16.63	
			1.35– 2.35	2.939	3.275	0.90	16.13	
			Average	2.797	3.131	0.90	15.40	
" 6	6.3	50 gm. glucose at 1.35.	3.17– 4.17	2.178	2.825	0.77	13.45	Standard diet Sept. 5 5 p.m.
			4.17– 5.17	2.372	3.003	0.79	14.40	
			5.17– 6.20	2.493	3.228	0.77	14.64	
			Average			0.78	14.18	
" 8	6.0	After 3 gm. Na_2CO_3 at 9.40.	10.36–11.36	2.087	2.620	0.80	12.58	Standard diet Sept. 7 5 p.m.
			11.36–12.37	2.078	2.812	0.74	13.30	
			12.37– 1.37	2.031	2.460	0.83	11.91	
			Average	2.085	2.631	0.79	12.59	
" 9	6.2	Basal.	10.06–11.06	2.341	3.505	0.67	16.43	Standard diet Sept. 8 5 p.m.
			11.06–12.06	2.022	2.149	0.94	10.65	
			12.06– 1.06	2.658	3.418	0.78	16.28	
			Average	2.340	3.024	0.79	14.44	

sion in the ice box for several weeks. At the end of this time it was filtered through cloth rendered alkaline, as already described, and administered. The two successes recorded are possibly confirmatory of the findings of Kleiner and Meltzer although it is impossible to state this positively until the authors reveal their method of preparing their pancreatic "emulsion." It is also possible that the failures which we are obliged to admit may be due to some variation in the mode of preparation of the pancreatic material, to the length of time the material was permitted to extract in the ice box, for example, or to the interval which elapsed between the time the pancreas was removed and treatment begun.

In view of the fact that we have had many failures to influence the respiratory quotient more than two or three points with alkali and glucose alone (Table V, page 491) on animals anatomically completely deprived of the pancreas, and in view of the much more striking effects to be seen presently on partially operated animals, our inclination at present is to look upon Dog 45, treated with alkali alone, as one which had some residual potential for oxidation of glucose, and that the alkali only restored a missing condition.

Partially Depancreatized Dogs.

Dog 37 was deprived of its pancreas on Aug. 3, 1915, only about 2 gm. of pancreatic tissue being left around the duct. The method of procedure, in brief, was as follows: The basal metabolism was determined for two or more hourly periods on different days and also preliminary to the administration of glucose or carbonate or both. Having determined the basal metabolism, 50 gm. of glucose were given by stomach tube (if not promptly taken by the dog when offered with the food) and the respiratory exchange determined for several hourly periods. On subsequent days 50 gm. of glucose and 3 gm. of sodium carbonate or 3 gm. of carbonate alone were given *per os* and the previous procedure repeated. The results are given in Table VIII.

It will be noted that from Aug. 9 to Sept. 9 the basal metabolism as measured by the oxygen consumption remained practically the same; likewise the basal respiratory quotient. Nevertheless, while on Aug. 23 the administration of 50 gm. of glucose was followed by an average rise in quotient of eleven points, the administration of an equal quantity of glucose on Sept. 6 produced an increase in quotient of but two points and that in only one period. The administration of carbonate along with the glucose raised the respiratory quotient on Aug. 28 to an average of 0.94, or seventeen points above the basal level, and on Sept. 4 to 0.90, or thirteen points above the basal level, although the administration of carbonate alone, both on Aug. 31 and Sept. 8, produced no effect on the respiratory quotient. It is worthy of note that the total heat production after carbonate was lower than the basal metabolism. To sum up the observations on this dog, we may say that a partially depancreatized dog fed on "standard diet" (consisting of 150 gm. of lean meat, 30 gm. of lard, and 50 gm. of cracker meal) with the occasional addition of 50 gm. of glucose showed a gradual loss in ability to oxidize glucose, and that this function was decidedly improved in each instance by the oral administration of sodium carbonate with the glucose. The fact that the administration of carbonate alone was followed neither by a rise in quotient nor by an increase in carbon dioxide elimination per hour makes us believe that the rise in quotient after carbonate and sugar can mean only increased glucose oxidation.

TABLE IX.

Dog 42. Control of Effect of Na_2CO_3 on the Normal Dog.

Date.	Food, etc.	Period.	Respiratory metabolism.		
			CO_2	O_2	R. Q.
1916			*liters*	*liters*	
Sept. 22	Standard diet* previous day.	1	3.787	5.103	0.74
		2	2.912	3.878	0.75
" 23	Standard diet previous day 5 p.m.	1	3.032	3.941	0.77
		2	3.188	4.271	0.75
		3	3.317	4.477	0.74
" 24	Standard diet and 6 gm. Na_2CO_3 evening before. 200 cc. 1 per cent Na_2CO_3 just before experiment.	1	4.066	5.411	0.75
		2	3.761	4.820	0.78
		3	2.869	3.445	0.79†

* Beef heart, lard, and cracker meal. † Note lower CO_2.

In spite of these carefully controlled observations, it might still be contended, as intimated in the first part of this paper, that the rise in quotient after administration of combined glucose and carbonate was due to liberation of carbon dioxide from the salt by the hydrochloric acid of the gastric juice or that the carbonate might itself give off carbon dioxide while circulating in the blood. To satisfy ourselves that such was not the case, two experiments were done on normal dogs, Nos. 42 and 43. In one of these experiments the basal metabolism was determined after 24 and again after 72 hours of fasting, and then 2.5 to 5 gm. of sodium carbonate were given by mouth and the respiratory exchange determined for several hourly periods. As seen in the

TABLE X.

Dog 43. Control of Effect of Na_2CO_3 on Normal Dog.

Date.	Previous diet.	Experiment.	Period.	Respiratory metabolism.			N per hr.	Calories per hr.
				CO_2	O_2	R. Q.		
1915				*liters*	*liters*			
Sept. 28	Fasting 24 hrs.	Basal.	1	3.056	4.185	0.73	0.166	19.42
			2	3.076	4.284	0.72	0.166	19.88
			3	2.975	3.928	0.76	0.166	18.30
			Average	3.035	4.132	0.74		19.20
" 29	Fasting 48 hrs. after 5 gm. Na_2CO_3 the evening before and 2 gm. Na_2CO_3 in 300 cc. water just before the experiment.							
			1	3.294	4.502	0.73	0.194	20.90
			2	3.209	4.017	0.80	0.194	18.93
			3	3.142	4.460	0.70	0.194	20.67
			Average	3.215	4.326	0.74		20.17
" 30	Fasting 72 hrs.		1	3.434	4.424	0.78	0.142	20.86
			2	3.522	5.010	0.70	0.142	23.32
			3	3.088	3.818	0.81	0.142	18.11
			Average	3.348	4.417	0.76		21.09
Oct. 1	Fasting.		1	2.976	3.922	0.76		
			2	3.000	3.919	0.77		
" 4	"		1	2.718	3.580	0.78		
			2	2.538	3.409	0.74		
	2.5 gm. Na CO_3 in 400 cc. water.							
			1 and 2	6.246	8.102	0.77		
			3	3.561	4.655	0.76		

table, no appreciable effect, either on the amount of carbon dioxide eliminated, or on the respiratory quotient, was obtained. An experiment on Dog 42 that received the standard diet gave equally negative results.

Dog 57 was depancreatized on Aug. 21, 1916. A fragment weighing about 1.5 gm. was left around the main duct. 200 gm. of lean meat were given daily. On Sept. 2 a study of the basal metabolism gave quotients of 0.67 and 0.68 in successive periods, indicating that ability to oxidize glucose was completely lost. This was confirmed by the fact that after the administration of 50 gm. of glucose the respiratory quotient rose but two points. The next morning 200 cc. of 1 per cent sodium carbonate solution was administered intravenously and the respiratory exchange studied for two periods. The respiratory quotient rose to 0.72 during the first period but during the second period dropped to 0.59, which was found to be due to a leak in a valve. After the administration of 50 gm. of glucose and about 5¼ hours after the injection of the alkali, the respiratory quotient rose to 0.88 in the first, and 0.96 in the third period, both of which periods were obtained with the same set of absorbers. The low quotient of 0.65 was undoubtedly due to a leak.

TABLE XI.

Dog 57. Female. Weight 13 Kg. Partial Pancreatectomy, About 1.5 Gm. of Pancreas Left around the Main Duct.

Date.	Time.	Weight.	Respiratory metabolism.			Remarks.
			CO_2	O_2	R. Q.	
1915		*kg.*	*liters*	*liters*		
Sept. 2	4.09- 4.54	11.35	3.24	4.93	0.67	Basal.
	4.54- 5.58		4.94	7.10	0.68	
	6.05					50 gm. glucose *per os.*
	8.15- 8.55		2.97	4.22	0.70	
" 3	11.15-11.45					200 cc. of 1 per cent Na_2CO_3 intravenously.
	1.16- 2.02	10.75	4.62	6.39	0.72	
	2.03- 2.32		1.79	3.12	0.59	Valve leaked.
	4.15					50 gm. glucose *per os.*
	4.32- 5.16		2.75	3.13	0.88	
	5.16- 5.59		2.995	4.61	0.65	Valve leaked.
	5.59- 6.44		4.04	4.10	0.96	

Thus, the administration of glucose or carbonate alone caused but a slight rise in the respiratory quotient, while the administration of glucose at a time when the urine would otherwise have been sugar-free and when the carbonate must still have been circulating in the blood, as shown in previous experiments, in the first paper of this series, yielded a very significant increase in the respiratory quotient. This dog, anatomically speaking, still had some pancreas, but functionally, as indicated by the respiratory quotient, had completely lost the ability to oxidize sugar. It is worthy of note here that the observations on Dog 37 are confirmed by this experiment in which the alkali was injected directly into the blood and hence could not have come in contact with the hydrochloric acid of the gastric juice; also that Dog 57 received 200 gm. of lean beef daily. It is, we believe, a significant fact that both these partially depancreatized dogs seem to have lost their power to burn glucose to a large extent, while subsisting on a diet containing meat.

DISCUSSION.

We shall not attempt at this time a full explanation of the facts presented in this series of papers; but it will be advantageous to focus attention upon the most important facts that have been established beyond a reasonable doubt.

1. In completely depancreatized dogs the administration of sodium carbonate, whether by mouth or by the intravenous route, is promptly followed by a retention of considerable amounts of glucose of endogenous or exogenous origin. The retention is rarely, if ever, accompanied by any evidence of improved oxidation of glucose.
2. In partially depancreatized dogs which still have some capacity to oxidize glucose, the administration of sodium carbonate or sodium hydroxide with glucose is followed by a greater oxidation of glucose than when either is given alone.
3. Sodium carbonate itself causes but a slight increase in respiratory quotient, or none, whether administered *per os* or intravenously, to normal or diabetic dogs.

Thus, it seems that while sodium carbonate or sodium hydroxide alone cannot restore the lost function to an organism completely deprived of its ability to oxidize glucose, either substance can improve the capacity of the organism as a whole to oxidize glucose when this function is more or less crippled although not completely lost.

The importance of alkalies, particularly sodium carbonate and the alkaline sodium phosphates, in glycolysis (11) is well known. Rona and Döblin (12), as well as Rona and Arnheim (13), have called attention to the fact that the addition of phosphates or carbonates to blood increases its glycolytic power *in vitro*. More recently, Levene and Meyer (14) were able to demonstrate a conversion of glucose into lactic acid by leukocytes in the presence of an alkaline phosphate mixture under aseptic conditions. Thus, the ability of alkalies, particularly sodium carbonate and the alkaline sodium phosphates, to facilitate a primary cleavage of glucose by at least one body tissue, the blood, seems conclusively established. The manner in which this is brought about is as yet imperfectly understood.

Warburg (15) has shown that sodium hydroxide greatly increases the oxidation of fertilized eggs of the sea urchin although it does not enter the egg. Osterhout (16) concludes that "small amounts of NaOH are able to produce a considerable increase of permeability" and calls attention to the fact that "increasing the permeability of the egg for products of oxidation would increase oxidation." Loeb and Wasteneys (17) confirmed the observations of Warburg, but found that with *Arbacia* eggs ammonium hydroxide also increased the rate of oxidation. "The fact that NaOH acts more powerfully than NH_4OH" in the same concentration of substance, they believe, "excludes the idea that we are dealing here with an effect of the undissociated molecule, and the parallelism in the effects of the two bases suggests a common source of their action;" namely, the OH ion. "The fact that the weak base acts more powerfully than we should expect from its degree of dissociation," may be ascribed, they think, to its greater power of penetration into the egg. "In this case one can explain all the phenomena on the assumption that while the greater part of oxygen is consumed at the surface of the egg, some diffuses into the egg and is consumed in the layers adjacent to the surface."

In view of the lack of definite knowledge as to the conditions under which the cleavage of the glucose molecule is brought about or the manner in which this process takes place, any attempt to describe the mechanism whereby the oxidation of glucose in the depancreatized dog is accelerated must, for the present, be mere speculation. It might be suggested that sodium hydroxide or sodium carbonate accelerates the oxidation of sugar, by increasing the permeability of the cell for oxygen or glucose or for both, or that the union of the alkaline salt with the sugar forms a combination that can be fixed at the surface of the cell and there oxidized. On the other hand, the alkali may act by creating a more favorable environment for the glycolytic action of a cell which still possesses the potential capacity to burn glucose.

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INDEX TO VOLUME XXVII.

Addis, T., and Watanabe, C. K. The rate of urea excretion. II. The rate of excretion of administered urea in young healthy adults on a constant diet, 249

——, and ——. The volume of urine in young healthy adults on a constant diet, 267

Acetone in urine, colorimetric method for determination of, 209

Acid and alkali, influence on glycosuria and hyperglycemia, 481

——, isobutyric, action of hydrogen peroxide on homologues of, 433

Acidity of plant cells shown by natural indicators, 233

Acidosis and carbohydrate metabolism, relationship of creatinuria to, 151

—— and creatine elimination, possible interrelations between, 127

Acids and alkalies, permeability to, of living cells, 225

——, amino-, of blood serum, influence of ether anesthesia on, 45

——, amino-, and carbohydrates, reaction between, as probable cause of humin formation, 71

——, fatty, branched chain, oxidation of, 433

—— and potassium salts, analogy for, of mechanism of diffusion (of electrolytes through membranes of living cells), 363

Alkali and acid, influence on glycosuria and hyperglycemia, 481

—— blue in organism, 377

Alkali, influence on creatine elimination during inanition, 141

——, influence on creatinuria of phlorhizin glycosuria, 147

——, influence on respiratory metabolism after total and partial pancreatectomy, 517

Alkalies and acids, permeability to, of living cells, 225

Allen, C. H. See Levene and Allen, 433

Amino-acids of blood serum, influence of ether anesthesia on, 45

—— and carbohydrates, reaction between, as probable cause of humin formation, 71

Ammonia and urea in muscle, direct determination of, 95

Anesthesia, ether, influence on amino-acids of blood serum, 45

Autolysis, influence of bile on, 243

Baumann, Emil J. See Underhill and Baumann, 147, 151, 169.

Bengis, Robert. See Salant and Bengis, 403

Bile, influence of, on autolysis, 243

Blood fat and blood sugar content of dogs under influence of hydrazine, interrelations of, 169

—— of goats, changes in cholesterol content of, following cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment and subsequent castration, 273

Blood serum, influence of ether anesthesia on amino-acids of, 45
——, small amounts of, separate determination of cholesterol and cholesterol esters in, 107
—— sugar and blood fat content of dogs under influence of hydrazine, interrelations of, 169
Bloor, W. R., and Knudson, Arthur. The separate determination of cholesterol and cholesterol esters in small amounts of blood, 107
Bodansky, A. The chymase of *Solanum elaeagnifolium*, 103
Bogert, L. Jean. See Underhill and Bogert, 161
Bread, white, yeast, and polished rice, nutritive value of, determined by experiments on man, 173
Butter fat and lard in growth, comparative value of, 51

CALORIMETRY, clinical, 217
Carbohydrate metabolism and acidosis, relationship of creatinuria to, 151
Carbohydrates and amino-acids, reaction between, as probable cause of humin formation, 71
Carbonate of sodium administered to depancreatized dogs, is glucose retained deposited as glycogen, after, 499
Carotin, toxicity of, 213
Carotinoids of plant, and of cow, horse, sheep, goat, pig, and hen, physiological relation of, 27
Castration following cholesterol feeding combined with Roentgen treatment, changes in cholesterol content of blood of goats after, 273
Cells, living, mechanism of diffusion of electrolytes through membranes of, 339, 353, 363
Cells, living, permeability of, to acids and alkalies, 225
—— of plant, acidity of, shown by natural indicators, 233
Chloride, potassium, diffusion of, out of egg of *Fundulus*, and relative efficiency of different ions for salt effect, 353
Cholesterol absorption, mechanism of, 463
—— added to diet deficient in fats, influence on growth of white mouse, 393
—— and cholesterol esters, separate determination of, in small amounts of blood, 107
—— content of blood of goats, changes in, following cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment and subsequent castration, 273
Chymase of *Solanum elaeagnifolium*, 103
Coal tar colors, physiological and pharmacological studies on, 403
Colorimetric method for determination of acetone in urine, 209
Colors, coal tar, physiological and pharmacological studies on, 403
Corn, nutritive properties of, 193
Cottonseed meal as an incomplete food, 15
Creatine elimination and acidosis, possible interrelations between, 127
—— elimination during inanition, influence of alkali upon, 141
—— metabolism, studies in, 127, 141, 147, 151
Creatinuria of phlorhizin glycosuria, influence of alkali upon, 147
——, relationship of, to carbohydrate metabolism and acidosis, 151

CSONKA, FRANK A. A colorimetric method for the determination of acetone in urine, 209

DESICCATOR, vacuum, electrically heated, 429

Determination of acetone in urine, colorimetric method for, 209

——, direct, of urea and ammonia in muscle, 95

—— of nitric nitrogen, method for, 327

——, separate, of cholesterol and cholesterol esters in small amounts of blood, 107

Diabetes, pancreatic, in the dog, 481, 499, 517

Diet, changes in, determining alterations in output of urinary constituents, 161

——, constant, urine volume in young healthy adults on, 267

—— deficient in fats and same diet with cholesterol added, influence on growth of white mouse, 393

——, rate of excretion of administered urea in young healthy adults on constant diet, 249

——, relation of unidentified factors (fat-soluble *A* and water-soluble *B*) of, to growth-promoting properties of milk, 33

Dietary conditions bearing on problem of growth in rats, 1

DU BOIS, EUGENE F. See GEPHART, DU BOIS, and LUSK, 217

Dyes, fat-soluble, experiments with, 403

ECKLES, C. H., and PALMER, LEROY S. The influence of parturition on the composition and properties of the milk and milk fat of the cow, 313

EDDY, WALTER H. The isolation of a growth-producing substance from sheep pancreas, 113

Egg of *Fundulus*, diffusion of KCl out of, and relative efficiency of different ions for salt effect, 353

Elaeagnifolium, *Solanum*, chymase of, 103

Electrolytes, mechanism of diffusion of, through membranes of living cells, 339, 353, 363

Esters of cholesterol, and cholesterol, separate determination of, in small amounts of blood, 107

Ether anesthesia, influence on amino-acids of blood serum, 45

Evolution and nutrition, 309

EWING, P. V. See WELLS and EWING, 15

FAT of blood and blood sugar content of dogs under influence of hydrazine, interrelations of, 169

—— of butter, and lard, in growth, comparative value of, 51

—— of cow's milk, influence of parturition on composition and properties of, 313

Fat-soluble *A* and water-soluble *B*, unidentified factors of diet, relation of, to growth-promoting properties of milk, 33

—— dyes, experiments with, 403

Fats, diet deficient in, and with cholesterol added, influence on growth of white mouse, 393

Fatty acids, branched chain, oxidation of, 433

FENGER, FREDERIC. Phosphatides in the ductless glands, 303

Food, incomplete, cottonseed meal as, 15

Fundulus, egg of, diffusion of KCl out of, and relative efficiency of different ions for salt effect, 353

FUNK, CASIMIR. The study of certain dietary conditions bearing on the problem of growth in rats, 1

FUNK, CASIMIR, LYLE, W. G., and McCASKEY, DONALD. The nutritive value of yeast, polished rice, and white bread, as determined by experiments on man, 173
——, and MACALLUM, ARCHIBALD BRUCE. Studies on growth. III. The comparative value of lard and butter fat in growth, 51. IV. The action of yeast fractions on the growth of rats, 63

GEPHART, FRANK C., DU BOIS, EUGENE F., and LUSK, GRAHAM. Clinical calorimetry. XVIII. The number of places of significant figures in the data of metabolism experiments, 217
Glands, ductless, phosphatides in, 303
Glucose retained when sodium carbonate is administered to depancreatized dogs, deposited as glycogen, 499
Glycogen, glucose deposited as, after sodium carbonate is administered to depancreatized dogs, 499
Glycosuria and hyperglycemia, influence of alkali and acid upon, 481
——, phlorhizin, influence of alkali upon creatinuria of, 147
Growth, comparative value of lard and butter fat in, 51
——, experimental studies on, 393
Growth-producing substance, isolation of, from sheep pancreas, 113
Growth-promoting properties of milk, relation of, to unidentified factors of diet, fat-soluble *A* and water-soluble *B*, 33
Growth of rats, action of yeast fractions on, 63
—— in rats, dietary conditions bearing on the problem of, 1
Growth, studies on, 51, 63
—— of white mouse on diet deficient in fats and same diet with cholesterol added, 393

HAAS, A. R. The acidity of plant cells as shown by natural indicators, 233
——. The permeability of living cells to acids and alkalies, 225
HEDENBURG, O. F. See WELLS and HEDENBURG, 213
HOGAN, ALBERT G. The nutritive properties of corn, 193
Humin formation, reaction between amino-acids and carbohydrates as probable cause of, 71
Hydrazine, interrelations of blood fat and blood sugar content of dogs under influence of, 169
Hydrogen ion concentration, relation of changes in, to oxidase reactions, 299
—— peroxide, action on homologues of isobutyric acid, 433

ION concentration, hydrogen, relation of changes in, to oxidase reactions, 299
Ions, relative efficiency of, for salt effect, and diffusion of KCl out of egg of *Fundulus*, 353
Isobutyric acid, action of hydrogen peroxide on homologues of, 433

KNUDSON, ARTHUR. See BLOOR and KNUDSON, 107
KRAMER, B., MARKER, J., and MURLIN, J. R. Pancreatic diabetes in the dog. II. Is the glucose retained when sodium carbonate is administered to depancreatized dogs deposited as glycogen, 499
——. See MURLIN and KRAMER, 481, 517

KURIYAMA, SHIGENOBU. The fate of alkali blue in the organism, 377

LARD and butter fat in growth, comparative value of, 51

LEVENE, P. A., and ALLEN, C. H. The oxidation of branched chain fatty acids. I. The action of hydrogen peroxide on the homologues of isobutyric acid, 433

LOEB, JACQUES. The mechanism of the diffusion of electrolytes through the membranes of living cells. I. The necessity of a general salt effect upon the membrane as a prerequisite for this diffusion, 339. II. The diffusion of KCl out of the egg of *Fundulus* and the relative efficiency of different ions for the salt effect, 353. III. The analogy of the mechanism of the diffusion for acids and potassium salts, 363

——, and NORTHROP, J. H. Nutrition and evolution, 309.

LUDEN, GEORGINE. Observations on the changes in the cholesterol content of the blood of goats, following cholesterol feeding alone, Roentgen treatment alone, and cholesterol feeding combined with Roentgen treatment and subsequent castration, 273

LUSK, GRAHAM. See GEPHART, DU BOIS, and LUSK, 217

LYLE, W. G. See FUNK, LYLE, and MCCASKEY, 173

MACALLUM, ARCHIBALD BRUCE. See FUNK and MACALLUM, 51, 63

MARKER, J. See KRAMER, MARKER, and MURLIN, 499

MCCASKEY, DONALD. See FUNK, LYLE, and MCCASKEY, 173

MCCOLLUM, E. V., SIMMONDS, N., and PITZ, W. The relation of the unidentified dietary factors, the fat-soluble *A*, and water-soluble *B*, of the diet to the growth-promoting properties of milk, 33

Membranes of living cells, mechanism of diffusion of electrolytes through, 339, 353, 363

Metabolism, carbohydrate, and acidosis, relationship of creatinuria to, 151

—— of creatine, studies in, 127, 141, 147, 151

—— experiments, number of places of significant figures in, 217

——, respiratory, influence of alkali on, after total and partial pancreatectomy, 517

Milk and milk fat of cow, influence of parturition on composition and properties of, 313

——, relation of growth-promoting properties of, to unidentified factors of diet, fat-soluble *A* and water-soluble *B*, 33

MUELLER, J. HOWARD. The mechanism of cholesterol absorption, 463

MURLIN, J. R., and KRAMER, B Pancreatic diabetes in the dog. I. The influence of alkali and acid upon glycosuria and hyperglycemia, 481 III. The influence of alkali on the respiratory metabolism after total and partial pancreatectomy, 517

——. See KRAMER, MARKER, and MURLIN, 499

Muscle, direct determination of urea and ammonia in, 95

NITROGEN, nitric, method for determination of, 327

Northrop, J. H. See Loeb and Northrop, 309

Nutrition and evolution, 309

Nutritive properties of corn, 193

OXIDASE reactions, relation to changes in hydrogen ion concentration, 299

Oxidation of branched chain fatty acids, 433

PALMER, Leroy S. The physiological relation of plant carotinoids to the carotinoids of the cow, horse, sheep, goat, pig, and hen, 27

——. See Eckles and Palmer, 313

Pancreas of sheep, isolation of growth-producing substance from, 113

Pancreatectomy, total and partial, influence of alkali on respiratory metabolism after, 517

Pancreatic diabetes in the dog, 481, 499, 517

Parturition, influence on composition and properties of milk and milk fat of cow, 313

Permeability of living cells to acids and alkalies, 225

Peroxide of hydrogen, action on homologues of isobutyric acid, 433

Pharmacological and physiological studies on coal tar colors, 403

Phlorhizin glycosuria, creatinuria of, influence of alkali upon, 147

Phosphatides in the ductless glands, 303

Physiological and pharmacological studies on coal tar colors, 403

—— relation of plant carotinoids to carotinoids of cow, horse, sheep, goat, pig, and hen, 27

Pitz, W. See McCollum, Simmonds, and Pitz, 33

Plant cells, acidity of, shown by natural indicators, 233

Potassium chloride, diffusion of, out of egg of *Fundulus*, and relative efficiency of different ions for salt effect, 353

—— salts, and acids, analogy for, of mechanism of diffusion (of electrolytes through membranes of living cells), 363

REED, Guilford B. The relation of oxidase reactions to changes in hydrogen ion concentration, 299

Rice, polished, yeast, and white bread, nutritive value of, determined by experiments on man, 173

Robertson, T. Brailsford. Experimental studies on growth. VIII. The influence of a diet deficient in fats, and of the same diet with cholesterol added, upon the growth of the white mouse, 393

——, and Schmidt, Carl L. A. An electrically heated vacuum desiccator, 429

Roentgen treatment alone and with cholesterol feeding, and subsequent castration, changes in cholesterol content of goat's blood after, 273

Ross, Ellison L. The influence of ether anesthesia in amino-acids of blood serum, 45

Roxas, M. L. The reaction between amino-acids and carbohydrates as a probable cause of humin formation, 71

SALANT, William, and Bengis, Robert. Physiological and pharmacological studies on coal tar colors. I. Experiments with fat-soluble dyes, 403

Salt effect upon membrane of living cells, necessity of, for diffusion of electrolytes, 339
—— effect, relative efficiency of different ions for, and diffusion of KCl out of egg of *Fundulus*, 353
Salts of potassium, and acids, analogy for, of mechanism of diffusion (of electrolytes through membranes of living cells), 363
Scales, F. M. A method for the determination of nitric nitrogen, 327
Schmidt, Carl L. A. See Robertson and Schmidt, 429
Serum of blood, influence of ether anesthesia on amino-acids of, 45
Simmonds, N. See McCollum, Simmonds, and Pitz, 33
Sodium carbonate administered to depancreatized dogs, is glucose retained deposited as glycogen, after, 499
Solanum elaeagnifolium, chymase of, 103
Sugar of blood and blood fat content of dogs under influence of hydrazine, interrelations of, 169
Sumner, James B. The direct determination of urea and ammonia in muscle, 95

TAR (coal) colors, physiological and pharmacological studies on, 403
Tatum, Arthur L. The influence of bile on autolysis, 243

UNDERHILL, Frank P. Studies in creatine metabolism. I. Possible interrelations between acidosis and creatine elimination, 127. II. The influence of alkali upon creatine elimination during inanition, 141.
——, and Baumann, Emil J. Studies in creatine metabolism. III. The influence of alkali upon the creatinuria of phlorhizin glycosuria, 147. IV. The relationship of creatinuria to carbohydrate metabolism and acidosis, 151
——,and—. The interrelations of blood fat and blood sugar content of dogs under the influence of hydrazine, 169
——, and Bogert, L. Jean. Alterations in the output of certain urinary constituents as determined by changes in the character of the diet, 161
Urea, administered, rate of excretion of, in young healthy adults on constant diet, 249
—— and ammonia in muscle, direct determination of, 95
—— excretion, rate of, 249
Urinary constituents, alterations in output of, determined by changes in character of diet, 161
Urine, acetone in, colorimetric method for determination of, 209
—— volume in young healthy adults on constant diet, 267

WATANABE, C. K. See Addis and Watanabe, 249, 267
Water-soluble *B* and fat-soluble *A*, unidentified factors of diet, relation of, to growth-promoting properties of milk, 33
Wells, C. A., and Ewing, P. V. Cottonseed meal as an incomplete food, 15
Wells, H. Gideon, and Hedenburg, O. F. The toxicity of carotin, 213

YEAST fractions, action of, on growth of rats, 63
——, polished rice, and white bread, nutritive value of, determined by experiments on man, 173

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